To my beloved grandparents, who were always proud of me.

Precise knowledge of self and precise knowledge of the threat leads to victory.

-The Art of War, by Sun Zi.

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Global switches and fine-tuning: the multifaceted role of plant hormones in regulating rice immunity against the leaf blight pathogen *Xanthomonas oryzae* pv. *oryzae*

Jing XU

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences

Dutch translation of the title:

Globale regulatoren en fijnregeling: de veelzijdige rol van plantenhormonen in de resistentie van rijst tegen de bladpathogeen *Xanthomonas oryzae* pv. *oryzae*.

Cover illustration:

Interpretation of the battlefield where plant hormones, rice and *Xanthomonas oryzae* pv. *oryzae* are the players.

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Contents

List of Abbreviations

ABA: abscisic acid

AHLs: *N*-acyl homoserine lactones

AI: autoinducer

BLB: bacterial leaf blight

BR: brassinosteroid

BTH: benzothiadiazole S-methylester

CK: cytokinin

DAMPs: damage-associated molecular patterns

DF: diffusible factor

DSF: diffusible signal factor

EPS: extracellular polysaccharides

ET: ethylene

ETI: effector-triggered immunity

ETS: effector-triggered susceptibility

Flu: fluridone

GAs: gibberellins

HR: hypersensitive response

IAA: indole-3-acetic acid

IAAsp: indole-3-acetylaspartic acid

IPT: isopentenyl transferase

ISR: induced systemic resistance

JA: jasmonic acid

LPS: lipopolysaccharides

LRR: leucine-rich repeat

M(A)PK: mitogen-activated protein kinase

NahG: salicylate hydroxylase

NBS: nucleotide-binding site

NIL: near-isogenic line

NO: nitric oxide

NPR1: non-expresser of PR proteins 1

PAMP: pathogen-associated molecular pattern

PTI: PAMP-triggered immunity

PR: pathogenesis-related

PRR: pattern recognition receptor

Pst: *Pseudomonas syringae* pv. *tomato*

QS: quorum sensing

R: resistance

ROS: reactive oxygen species

RSM: rice signal molecule

SA: salicylic acid

SAR: systemic acquired resistance

TALE: transcription activator-like effectors

TF: transcription factors

TOR: target of rapamycin

SLR1: slender rice1

T2SS: type II secretion system

T3SS: type III secretion system

Xcc: *Xanthomonas campestris* pv. *campestris*

Xoo: *Xanthomonas oryzae* pv. *oryzae*

Chapter 1

Problem statement and thesis outline

1.1 Problem statement

As one of the world's most important staple foods, rice provides more than 20% of the daily calories for more than 3.5 billion people worldwide, many of whom are living in poverty (www.irri.com). Since rice easily adapts to the surrounding environment, it can be grown in a wide range of agricultural contexts and is productive in many situations where other crops would fail. Today, rice is cultivated in more than one hundred countries. In 1962, 228.104 million tons of rice were produced on approximately 120 million hectares of paddy field (1.91 tons/Hectare). During the last decades, however, major progress has been made in enhancing rice productivity. As a result, world rice production has almost tripled, reaching 697.225 million tons and 158 million hectares by 2012 (4.40 tons/Hectare) (Table 1.1, data are generated from http://ricestat.irri.org:8080/wrs2/entrypoint.htm). This tremendous yet much needed increase in production was achieved through the adoption of improved high-yielding varieties, adequate irrigation, enhanced use of fertilizers and other complementary inputs. Yet, in recent years, population growth has outpaced rice production (Hossain 2007). Whereas the annual population growth continues to swell at a rate of approximately 1.14% per year, rice yields have stuck fast at around 6 tons per hectare in the countries accounting for 75% of the global rice output. If these trends continue, demand for rice in many parts of Asia will outstrip supply within a few years. Such lag in production will disproportionally affect low-income countries where people consume more rice and the population grows faster.

Table 1.1 World rice statistics from 1962 to 2012

Year	Paddy Production (million tons)	Yield (t/Ha)	Milled Rice (million tons)	Consumption (million tons)
1962	228.104	1.91	155.105	149.999
1972	306.231	2.31	208.937	212.976
1982	418.169	2.98	284.974	277.833
1992	524.181	3.58	354.003	355.574
2002	563.064	3.83	378.199	405.916
2012	697.225	4.40	467.601	466.092

Diseases caused by microbial pathogens have always been one of the major production constraints throughout rice growing history. On above ground plant parts, fungal diseases such as rice blast (*Magnaporthe oryzae*), sheath blight (*Rhizoctonia solani*) and brown spot (*Cochliobolus miyabeanus*) together with bacterial leaf blight (BLB) account for the highest yield losses. BLB is caused by the gram-negative bacterium *Xanthomonas oryzae* pv. o*ryzae* (*Xoo*) which can affect the stem, leaf sheath, leaf blade and panicle of the rice plant. Young transplanted seedlings are especially susceptible to the disease and often completely die soon after infection (Reddy et al. 1979). Yield losses of 10%-50% resulting from BLB have been reported, and if the disease develops early it can destroy up to 80% of a crop (Ou 1985). In Japan alone, annual losses are estimated to be between 22,000 and 110,000 tons. In addition to causing enormous yield losses, BLB also results in poor grain quality and a high proportion of broken kernels (http://www.knowledgebank.irri.org/decision-tools/rice-doctor/rice-doctor-fact-sheets/item/bacterial-blight).

Current control measures for BLB include a variety of cultural practices, chemical and biological control, disease forecasting, and most importantly, host plant resistance conferred by major *R* genes. The first key point is practicing field sanitation, such as removing old straw and weed hosts (Niño-Liu et al. 2006). Proper fertilization, especially avoiding an excess of nitrogen, is also important (Mew et al. 1993). In addition, some agrochemicals such as Agrimycin have been claimed to give good control of BLB (Khan et al. 2012; Singh et al. 1980). However, in addition to being environmentally undesirable and economically costly, chemical control of BLB in the tropical monsoon climate of Asia is impractical (Lee et al. 2003), and to date no truly effective bactericides are commercially available (Niño-Liu et al. 2006). In this context, biological control has been receiving increasing attention as an environmentally sound and cost-effective alternative to chemical BLB control (Gnanamanickam 2009). Yet, despite some biological agents showing promising results in laboratory trials, the effectiveness and robustness of biological control under field conditions is still questionable (Niño-Liu et al. 2006). Releasing resistant varieties that carry one or more major *R* genes thus remains the most effective approach for BLB control. Conditioning extremely high levels of disease resistance, *R*-gene controlled immunity is effective only against those pathogen strains carrying cognate avirulence proteins. Moreover, most resistant cultivars do not withstand more than one or two years of cultivation before succumbing to BLB due to either breakdown or gradual erosion of the resistance in face of the high variability of the pathogen population. Hence, there is considerable incentive to develop new control strategies providing efficient, long-lasting, broad-spectrum and environmentally friendly pathogen protection. Among these strategies, approaches

capitalizing on the plant's own defensive repertoire seem particularly promising (Song and Goodman 2001).

To resist their potential colonization by microbial pathogens and herbivorous insects, plants have evolved a plethora of constitutive and inducible defense mechanisms. Many of these responses are orchestrated by a network of cross-communicating signal transduction pathways within which plant hormones play key roles (Grant and Jones 2009; Pieterse et al. 2009). *Xoo* was recently elected as the fourth most important pathogenic bacterium worldwide (Mansfield et al. 2012), and the rice-*Xoo* pathosystem has emerged as a paradigm for investigation of foliar pathogenicity (Niño-Liu et al. 2006). However, despite the identification and characterization of several major *R* genes and their corresponding avirulence genes in the pathogen, surprisingly little is known about the host defense signaling circuitry controlling disease and resistance in rice-*Xoo* interactions. In view of this knowledge gap, the primary objective of this work was to further our fundamental understanding of hormone defense signaling in cereals, using the rice-*Xoo* interaction as a model system. More specifically, we sought to:

1. Unravel the tapestry of hormone signaling pathways governing susceptibility and resistance against *Xoo*.
2. Gain insight into the dynamic interplay and cross-communication between individual hormones and evaluate the impact of hormone crosstalk in shaping the outcome of rice-*Xoo* interactions.
3. Elucidate if and how *Xoo* manipulates the rice hormone network in order to antagonize effective defense responses.
4. Explore the possible role and importance of hormones in orchestrating rice-*Xoo* interkingdom signaling and assess if plant hormones affect bacterial virulence by tapping into the quorum sensing machinery of *Xoo*.

1.2 Thesis outline

This thesis starts with a concise literature review (**Chapter 2**) summarizing our current knowledge on the principles and basic mechanisms of plant innate immunity and bacterial virulence. In the first part of this chapter, we briefly review various aspects of the plant innate immune system, including the sensitive perception mechanisms by which plants recognize their attackers as well as the ensuing signal transduction and mobilization of defense responses, and briefly touch upon the role of plant hormones in fine-tuning plant defenses to

the type of attacker encountered. The second part introduces the biology of *Xoo* and its virulence factors, as well as the corresponding secretion systems. In addition, in **Chapter 3**, we survey the latest discoveries dealing with hormone-mediated immunity in rice, thereby focusing on the similarities and differences with findings in Arabidopsis. We highlight interactions between hormone signaling, rice defense and pathogen virulence and describe how detailed knowledge of hormone crosstalk mechanisms can be used for engineering durable disease resistance in cereal crops.

The following **chapters**, **4** and **5**, are dedicated to the effects of different plant hormones in orchestrating rice defense against *Xoo*. In **Chapter 4**, we investigate the role of the classic abiotic stress hormone abscisic acid (ABA) in compatible and incompatible rice-*Xoo* interactions and explore the underlying mechanisms. By combining exogenous hormone applications, time-resolved ABA measurements and bioassays using ABA-deficient and/or ABA-deficient plants, we show that ABA functions as a virulence factor of *Xoo*. Moreover, several lines of evidence indicate that this immune-suppressive effect of ABA is due at least in part to suppression of effective SA-mediated defenses. Resistance induced by the ABA biosynthesis inhibitor fluridone, however, appeared to operate in a SA-independent manner and is likely due to induction of non-specific physiological stress.

In **Chapter 5**, we characterize the role of the classic growth hormone cytokinin (CK) in the rice-*Xoo* interaction. Similar to ABA, endogenous CK levels strongly increase following infection with virulent *Xoo*, resulting in an enhanced state of susceptibility that favors bacterial growth and subsequent disease development. However, unlike ABA-mediated disease susceptibility, CK promotes *Xoo* infection independently of SA antagonism. Instead, combined pharmacological, proteomic and molecular findings suggest that CK activates the master growth-regulatory protein Target of Rapamycin (TOR), leading to suppression of basal immune responses that normally serve to limit *Xoo* growth.

Chapter 6 addresses a different aspect of plant defense signaling and investigates whether and how plant hormones are involved in interkingdom signaling and affect microbial virulence by interfering with bacterial cell-to-cell communication mechanisms. Consistent with its role in promoting BLB development, our results suggest that ABA enhances the swimming motility of *Xoo*. In contrast, SA inhibits swimming but induces production of extracellular polysaccharides (EPS) and the water-soluble pigment xanthomonadin. Moreover, we demonstrate that SA stimulates the biosynthesis of bacterial quorum sensing signals and present evidence suggesting that *Xoo* deploys the LuxR-like ‘solo’ protein OryR to ‘eavesdrop’ on plant ABA and SA titers. Finally, we propose two integrative models explaining the role and function of plant hormones and bacterial quorum sensing factors as

key messengers of reciprocal rice-*Xoo* signal interactions and discuss the importance of inter-kingdom communication in molding pathological outcomes.

Finally, in **Chapter 7**, we briefly summarize the main findings and discuss the practical implications and future prospects of the research conducted.

Chapter 2

General introduction

2.1 Plant immunity: principles and general mechanisms

Due to their sessile nature, plants are continuously threatened by a wide variety of microbial pathogens including bacteria, fungi, oomycetes and nematodes. Lacking the adaptive immunity that vertebrates rely on to respond to pathogens, plants have developed a complex set of sophisticated defense mechanisms to defend themselves against all these attackers (Nürnberger et al. 2004). Plant penetration by pathogens is achieved either by direct penetration of the plant surface or else by entry through wounds or natural openings, such as stomata, lenticels and hydathodes. To prevent or attenuate pathogen invasion, plants rely on a wide array of pre-formed physical and biochemical barriers that are effective against the large majority of potential attackers. However, despite the diversity of these constitutive defenses, some microbes succeed in overcoming or evading this pre-invasive layer of defense (Jones and Dangl 2006).

To impair further pathogen ingress, plants have evolved several mechanisms to recognize intruders and respond adequately by activating a concerted battery of post-invasive defenses. First, the primary immune response of plants recognizes conserved features of microbial pathogens, called either pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs). Several MAMPs have been identified thus far, including bacterial flagellin, lipopolysaccharides (LPS) and elongation factor Tu (EF-Tu), and fungal chitin and oomyceteous cellulose-binding elicitor proteins (Nürnberger 1999). MAMPs or PAMPs are detected by means of high-affinity membrane-bound receptor proteins referred to as Pattern Recognition Receptors (PPRs). Showing structural and functional similarities with animal Toll-like receptors, PRRs typically consist of an extracellular leucine-rich repeat (LRR) domain and an intracellular kinase domain (Zipfel 2008). Perception of MAMPs by PRRs triggers a stereotypical set of physiological events, including bursts of reactive oxygen species (ROS), ethylene (ET) release and massive transcriptional reprogramming, resulting in a basal level of resistance termed PAMP-triggered immunity (PTI) (Monaghan and Zipfel 2012).

However, during the co-evolutionary arms race between plants and their intruders, successful pathogens have acquired the ability to counter PTI by injecting effector proteins in the host cells, resulting in effector-triggered susceptibility (ETS) (Raman et al. 2012). Pathogen effectors are directed to many host cell compartments where they presumably act on one or more targets to dampen, delay and impede PRR-induced responses. By contrast, if only one of the many effectors delivered by a given pathogen into the host is recognized by the action of a cognate resistance (R) protein, then the host triggers a rapid and high-amplitude output response that stops pathogen growth. This superimposed layer of defense

is variably termed effector-triggered immunity (ETI), R-gene-mediated resistance or gene-for-gene resistance. R genes typically encode intracellular receptor proteins that contain a nucleotide-binding site (NBS) and LRRs. Most effectors are detected indirectly by NBS-LRRs from modifications the virulence proteins inflict on host target proteins. However, some NB-LRRs also directly bind pathogen proteins (Dangl et al. 2013).

Although ETI and PTI are associated with a largely overlapping set of defenses, effector recognition often culminates in the programmed suicide of a limited number of challenged host cells, clearly delimited from the surrounding healthy tissue (da Cunha et al. 2006). This so-called hypersensitive response (HR) is thought to benefit the plant by restricting pathogen access to water and nutrients and is correlated with an integrated set of physiological and metabolic alterations that are instrumental in impeding further pathogen ingress, among which a burst of oxidative metabolism leading to the massive generation of ROS. The dynamic plant immune system can be nicely presented as a 'zig-zag' model (Jones and Dangl 2006) (Figure 2.1).

Intriguingly, triggering of local defense can also mount a long-distance immune response termed systemic acquired resistance (SAR), in which naive tissues become resistant to a broad spectrum of otherwise virulent pathogens. SAR induction is hallmarked by local and systemic increases in endogenously synthesized salicylic acid (SA) and expression of a large battery of defense-related genes, including those encoding pathogenesis-related (PR) proteins (Vlot et al. 2008; Durrant and Dong 2004; Grant and Lamb 2006). Selected strains of root-colonizing bacteria or fungi are able to induce a phenotypically similar form of induced resistance, coined induced systemic resistance (ISR) (Heil and Bostock 2002). In contrast to SAR, ISR often functions independently of SA but requires intact responsiveness to jasmonic acid (JA) and ethylene (ET). Moreover, unlike SAR, ISR-expressing tissues do not show continuous defense activation, but rather are sensitized to express basal defense responses faster and/or more strongly in response to pathogen attack (Choudhary et al. 2007). This phenomenon is known as priming and confers a broad-spectrum resistance with minimal impact on seed set and plant growth. Because defense responses are only activated when necessary, priming offers an elegant solution to the plant's trade-off dilemma between efficient disease control and fitness costs involved in defense mobilization.

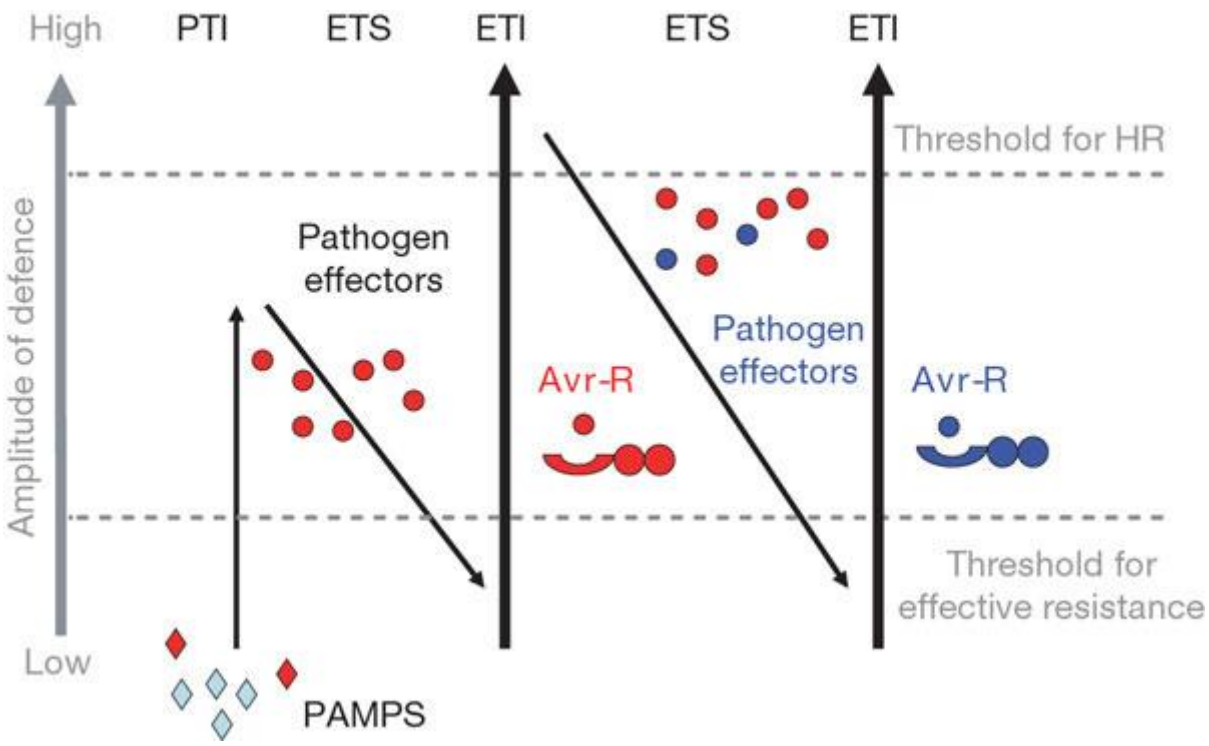

Figure 2.1 A zigzag model illustrates the quantitative output of the plant immune system (Jones and Dangl 2006)**.** PAMPs: pathogen-associated molecular patterns; PTI: PAMP-triggered immunity; ETS: effector-triggered susceptibility; ETI: effector-triggered immunity; HR: hypersensitive responses.

2.2 Hormonal modulation of plant innate immunity

Plant hormones such as SA, JA, ethylene (ET), abscisic acid (ABA), auxins, cytokinins (CKs), gibberellins (GAs) and brassinosteroids (BRs), have long been known to play pivotal roles in regulating plant growth, development and reproduction. Moreover, recent advances in plant stress research underpin the importance of hormones in orchestrating adaptive responses to biotic and abiotic challenges (Pieterse et al. 2009). Upon pathogen attack, plants synthesize a complex blend of hormones, thereby setting off a vast array of downstream immune responses, including the biosynthesis of antimicrobial secondary metabolites, stomatal closure, ROS accumulation, and local strengthening of plant cell walls by callose and lignin. In the dicot model plant *Arabidopsis thaliana*, SA, JA and ET are the archetypal immunity hormones and their importance in the hard wiring of the plant defense signaling network is well established. Although there are exceptions, SA is predominantly effective against biotrophic pathogens, whereas necrotrophic pathogens are usually deterred by JA/ET-driven defenses. Moreover, these two types of defenses often interact in a mutually antagonistic manner, suggesting that plant immunity follows in essence a binary model with SA and JA/ET having opposing influences. According to current concepts, other hormones, such as ABA, GA and auxin, influence plant immunity by modulating and feeding into the SA-JA-ET

backbone of the immune system (Pieterse et al. 2009). This so-called crosstalk between individual hormone pathways provides the plant with a powerful means to flexibly tailor its immune response to the type of attacking pathogen and to utilize its resources in a cost-efficient manner. However, exciting new developments demonstrate that this co-called crosstalk also enables successful pathogens to manipulate the plant's defense signaling network to their own benefit by shutting down effective defenses through negative network connections (Bari and Jones 2009; Pieterse et al. 2009; Melotto and Kunkel 2013).

Despite these paradigm-shifting advances, our current knowledge is limited by its heavy reliance on information derived from all but a few Arabidopsis-pathogen interactions and it is still largely unclear whether the conceptual framework emerging from these pathosystems will translate to other plant-microbe interactions. Recent studies using alternative plant models are now beginning to provide important additional insight into the immune-regulatory role of phytohormones. In rice, for instance, rapidly accumulating evidence indicates that disease resistance is controlled by a highly complex signaling circuitry that does not support a dichotomy between the lifestyle of a given pathogen and the effectiveness of SA, JA, and ET-mediated defenses (De Vleesschauwer, Gheysen, & Hofte, 2013). A more detailed description of the differences and similarities in defense networking in rice and Arabidopsis and the role of hormones herein will be presented in Chapter 3.

Having briefly reviewed the perception mechanisms by which plants recognize their attackers as well as the ensuing host immune responses and the regulation thereof, the next sections aim to distill current literature on the biology and virulence strategies of *Xanthomonas oryzae* pv. *oryzae*, causal organism of the devastating rice leaf blight (BLB) disease and a paradigm system for investigating foliar pathogenicity.

2.3 *Xanthomonas oryzae* pv. *oryzae* and its virulence determinants

2.3.1 Introduction about *Xanthomonas oryzae* pv. *oryzae* and bacterial leaf blight

2.3.1.1 The history and biology of *Xoo*

Bacterial leaf blight (BLB) disease was first reported in the Fukuoka Prefecture of Japan in 1884. Initially, BLB was believed to be caused by acidic soil. In 1909, Takaishi and coworkers succeeded in isolating bacteria from the dewdrops of diseased rice leaves and confirmed these bacteria to reproduce disease upon inoculation of healthy plants (Ou 1985).

Based on morphological and physiological characteristics, BLB-inflicting bacteria were consecutively classified as *Bacillus oryzae*, *Pseudomonas oryzae* and later *Xanthomonas oryzae*. In 1978, Dye reclassified the bacterium as *Xanthomonas campestris* pv. *oryzae* but after clearly distinguishing the species from the causal agent of bacterial leaf streak (BLS) disease, it was finally named as *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (Swings et al. 1990). The species resides within the family Xanthomonadaceae in the Gammaproteobacteria.

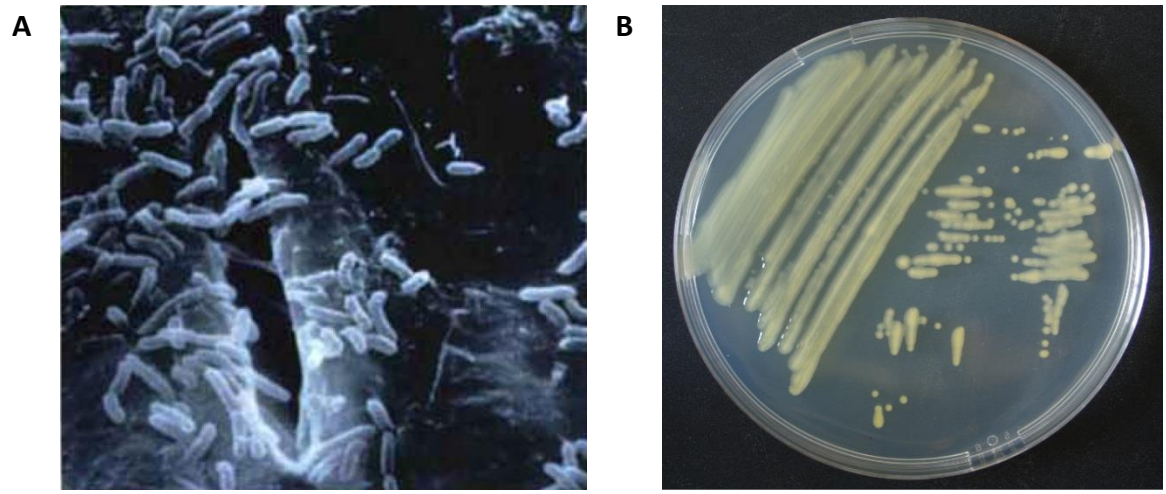

Figure 2.2 Morphology of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). A, *Xoo* cells surrounding the water pore on a rice leaf (Mew et al. 1993); B, Colonies of *Xoo* strain PXO99 on a peptone sucrose agrose (PSA) plate.

Xoo is a Gram negative, rod-shaped, round-ended species (Figure 2.2A) that is motile by means of a single polar flagellum. Individual cells are approximately 0.7-2 µm in length and 0.4-0.7 µm in width. *Xoo* is obligately aerobic and does not produce spores. The colonies on solid plates are normally round, mucoid, convex and yellow (Figure 2.2B). The optimal pH range for *Xoo* growth is 6-6.5, and optimal growth temperatures range from 25 to 30 °C.

2.3.1.2 The infection mode and distribution of *Xoo*

In natural circumstances, *Xoo* enters rice leaves through hydathodes at the leaf tip and leaf margin, multiplies in the epitheme until the logarithmic growth phase, and then soon moves to the xylem vessels, where active multiplication results in blight on the leaves. Wounds, especially new wounds, are also favourable entry sites for *Xoo*, enabling the bacteria to enter the xylem directly. Once inside the xylem, *Xoo* progresses through the leaf both vertically following the primary veins as well as laterally through commissural veins (Huang and De Cleene, 1989).

BLB symptoms typically begin with light green, water-soaked lesions anywhere along the leaf between the veins. Partly due to growth of opportunistic or saprophytic fungi, infected leaves eventually turn greyish white, and show wilting and rolling as they dry up and die (Figure 2.3A). At later stages of disease development, it is often difficult to distinguish BLB from other rice diseases, such as bacterial leaf streak caused by *Xanthomonas oryzae* pv. *oryzicola*. In the tropics, and especially on susceptible *indica* rice cultivars, *Xoo* also causes atypical blight symptoms referred to as 'kresek' and 'pale-yellow leaf'. Kresek occurs on young seedlings and may kill the entire plant in 2-3 weeks of time. Plants that survive kresek exhibit reduced tillering, stunted growth and an overall yellowish green color. At later stages of infection, excessive bacterial cells and extracellular polysaccharides (EPS) ooze out from hydathodes, forming beads or strands of exudate on the rice leaf surface (Figure 2.3B). This bacterial ooze not only serves as a diagnostic feature of BLB, but also constitutes a source of secondary inoculum (Ou, 1985).

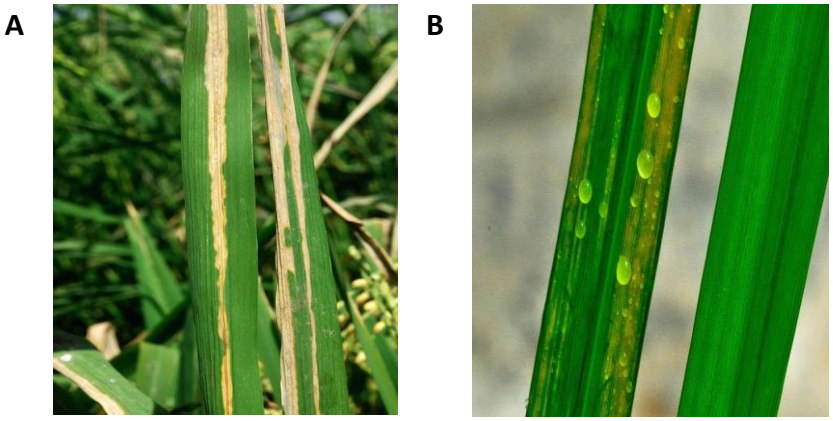

Figure 2.3 Diagnostic features of bacterial leaf blight (BLB) on rice. A, typical BLB lesions on rice leaves; B, *Xoo* ooze on the leaf surface. (Source of these two pictures: http://www.knowledgebank.irri.org/)

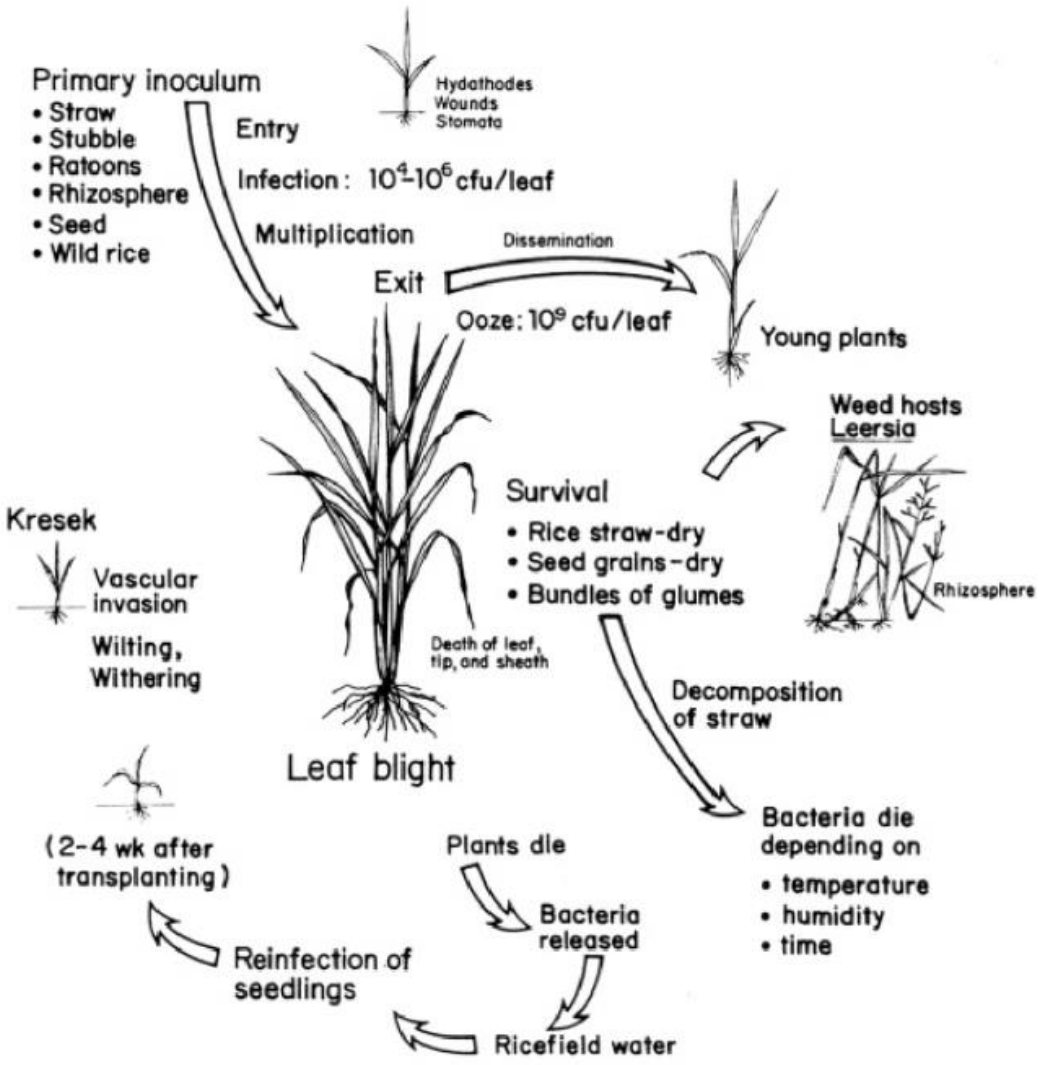

Figure 2.4 Epidemiology cycle of bacterial leaf blight (BLB) of rice (Alvarez et al, 1989).

The major primary inoculum source of BLB include bacteria from other infected rice plants and other hosts, and from contaminated rice stubble. In addition, bacteria existing in irrigation water, soil and seeds, are potential inoculum for BLB (Figure 2.4) (Alvares et al, 1989). Outbreaks of BLB often occur in compliance with typhoons, because the fierce winds, wind-blown rain and the wounds caused by them favor the distribution and penetration of *Xoo*.

2.3.2 Important virulence factors and strategies used by *Xoo*

Following plant entry, pathogens can either colonize xylem vessels or phloem sieve elements or live in inter- and intracellular spaces. To evade, overcome or attenuate host defense responses and to elicit the release of sufficient nutrients for colonization and proliferation,

pathogens deploy a diverse set of unique virulence strategies. These include the production of a broad arsenal of secreted virulence factors such as small effector proteins, low-molecular-weight phytotoxins, exopolysaccharides and cell wall degrading enzymes, as well as quorum sensing-mediated cell-to-cell communication mechanisms and disruption of plant hormone homeostasis.

2.3.2.1 Adhesion of *Xoo*

Bacterial attachment to biotic surfaces is mediated by specialized surface structures or macromolecules commonly known as adhesins. Adhesion is a highly conserved virulence mechanism among Gram-negative bacteria, and a key step in bacterial penetration and biofilm formation. Based on their assembly mechanism and structure, adhesins fall into two major classes: fimbrial and non-fimbrial adhesins. Fimbrial adhesins (or pili) are filamentous proteinaceous structures that are composed of multi-subunit heteropolymers and are assembled by proteins structurally related to type II secretion systems (Gerlach and Hensel 2007). In contrast, non-fimbrial adhesins consist of a single protein or homotrimers. Moreover, unlike fimbriae, most non-fimbrial adhesins are products of either two-partner or type V secretion systems, including the trimeric and non-trimeric autotransporters (Gerlach and Hensel 2007). Other non-fimbrial adhesins include small integral outer membrane adhesins and proteins secreted via type I secretion systems (Gerlach and Hensel 2007; Amano 2010).

Although much remains to be discovered about *Xoo* adhesion, it has been suggested that both fimbrial and non-fimbrial adhesins are important for full *Xoo* virulence. Genome analysis of several *Xoo* strains revealed the presence of several genes encoding adhesin-like proteins including XadA, XadB and YapH (Lee et al. 2005; Das et al. 2009; Ray et al. 2002). XadA is homologous to some of the best-characterized adhesins, including YadA of *Yersinia* spp. and UspA1 of *Moraxella catarrhalis* (Ray et al. 2002). XadB contains several domains similar to YadA, Hia autotransporter adhesin of *Haemophilus influenzae*, and *Burkholderia* autotransporter hemaglutinin-like protein, while YapH is the closest homolog of an autotransporter adhesin of *Yersinia* spp (Das et al. 2009). Both XadA and XadB play crucial roles in leaf attachment and entry following epiphytic survival, but are not required for wound infection. YadA, in contrast, is important but not indispensable for both epiphytic and wound infection. Recently, Pradhan et al (2012) reported a novel adhesin of *Xoo*, termed XadM. Like YadA, XadM plays an important role in both epiphytic and wound infection (Pradhan et al. 2012). Interestingly, XadM exhibits significant similarity to the enigmatic family of recombination hot spot proteins (RHS), making XadM the first RHS required for attachment and virulence in bacteria.

In addition to aforementioned adhesins that are widely conserved across the species, individual *Xoo* isolates may produce many other adhesin and adhesin-like proteins, often belonging to different subclasses. The reference strain PXO99A, for instance, is well known to express a set of filamentous hemagluttinin-like adhesins, the role of which in virulence remains to be identified (Salzberg et al. 2008). In addition, there are several genes in the *Xoo* genome predicted to be involved in the biosynthesis of type IV pili (Lee et al. 2005; Salzberg et al. 2008). A number of these genes have recently been implicated in *Xoo* virulence, including PilQ, a member of the type IV pili secretin proteins, which regulates twitching motility and biofilm formation (Das et al. 2009; Lim et al. 2008).

Biochemical and genetic analysis of these non-fimbrial adhesins and type IV pilus secretins revealed extensive functional redundancy as well as marked differences in adhesion characteristics of *Xoo* on the surface and inside of rice leaves. This molecular flexibility likely contributes to the ability of *Xoo* to adapt to different hosts or host tissues. Noteworthy in this respect is that the *xadM* knockout mutant exhibits lawn morphology due to reduced production of EPS (Pradhan et al. 2012). Moreover, it shows lower attachment efficiency towards nitrocellulose membranes coated with the polysaccharides xylan and cellulose, which mimics the rice cell wall.

2.3.2.2 Extracellular polysaccharides (EPS)

EPS are carbohydrate polymers secreted by bacteria that form either a tight capsule layer surrounding the bacterial cells or a loosely associated extracellular slime. Many phytopathogenic bacteria produce various EPS polymers during growth, and their importance in bacterial pathogenesis is well established (Denny 1995). EPS is believed to favor plant pathogens through multiple functions including: 1) providing protection from unfavorable environmental conditions as well as from toxic compounds encountered *in planta*; 2) causing wilting of host plants by blocking the water flow in xylem vessels; 3) facilitating absorption of water, minerals and nutrients; 4) contributing to biofilm formation, which is considered as a crucial process during early infection phases (Denny 1995).

Xanthomonas spp. produce copious amounts of an EPS known as xanthan gum. Xanthan is a high-molecular weight EPS polymer composed of repeating pentasaccharide units with a cellulose backbone and trisaccharide side chains (Sutherland 2001). *Xoo*-produced xanthan typically contains rhamnose, arabinose, galactose, glucose, mannose, xylose, galacturonic acid and glucuronic acid subunits, although the relative abundance of each may differ considerably among individual strains (Singh et al. 2006). The biosynthesis of xanthan

consists of three consecutive steps: 1) synthesis of nucleotidyl derivative precursors; 2) assembly of pentasaccharide subunits and addition of acetyl and pyruvate groups; and 3) polymerization of the pentasaccharide repeating units and secretion of xanthan (Ielpi et al. 1993). The final two steps are catalyzed by enzymes encoded by the *gum* gene cluster. In *Xoo*, this cluster consists of a tandem array of 12 open reading frames, which exhibit high similarity to the *gum* genes identified in *Xanthomonas campestris pv. campestris* (*Xcc*) (Yoon and Cho 2007). *Xoo* mutants impaired in xanthan production grew to lower levels *in planta* and elicited reduced disease symptoms on rice leaves, demonstrating the importance of xanthan in the pathogenesis of *Xoo* (Kim et al. 2009).

Xanthan was first suggested to promote bacterial fitness by forming a physical barrier that protects virulent *Xoo* from entrapment by fibrillar material (Huang and De Cleene, 1989). More recent data, however, also implicate xanthan in attachment and biofilm formation of *Xoo* (Das et al. 2009). Since BLB is a vascular disease, it is not inconceivable that the huge amounts of xanthan produced by virulent *Xoo* plug the vessels and block water flow, thus promoting leaf wilting and formation of water-soaked blight lesions. Supporting this assumption, Vidhyasekaran et al (1989) showed that treatment of rice roots with EPS triggers membrane leakage and wilting symptoms in aboveground plant parts (Vidhyasekaran et al. 1989). According to Singh et al., (2006), the compositional differences of xanthan gum produced by different *Xoo* strains play an important role in host specificity and may at least in part determine the pathogen's spectrum of virulence (Singh et al. 2006).

2.3.2.3 Extracellular enzymes and the type II secretion system (T2SS)

Apart from providing structural support, the plant cell wall also functions as a physical barrier blocking pathogen invasion and spread within infected tissues. Plant cell walls are composed of complex carbohydrate polymers including cellulose, hemicellulose and pectin, all of which are potential carbon sources for plant pathogens (Burke et al. 1974). Not surprisingly, therefore, many pathogens are known to secrete a vast array of cell-wall-degrading enzymes, including cellulases, xylanases, proteases and pectinases, to soften or breakdown plant cell walls, thereby facilitating pathogen entry and promoting leakage of nutrients for pathogen growth (Melotto and Kunkel 2013).

Xylan is a major component of plant cell walls, accounting for up to 60% of total cell wall carbohydrate content (Takeuchi et al. 1994). Rajeshwari et al (2005) showed that the xylanase-deficient mutant *xynB* is less virulent than the wild type strain, confirming the importance of xylanase in maintaining pathogenicity of *Xoo* (Rajeshwari et al. 2005). In

addition, lipases/esterases which hydrolyze acetyl ester and phenolic ester bonds in xylan, also contribute to the pathogenicity of *Xoo* (Aparna et al. 2009; Jha et al. 2007; Rajeshwari et al. 2005). Cellulases likewise play important roles in *Xoo* virulence. Recent genome analysis of *Xoo* strain KACC10331 revealed the presence of 12 genes encoding cellulases (Temuujin et al. 2011; Wang et al. 2008). Among these, EglXoA, EglXoB and CelXoB are absolutely required for *Xoo* pathogenicity, CelbXoA, BglXoB and BglXoF are important but not vital, whereas others appear to be dispensable (Table 2.1) (Hu et al. 2007; Temuujin et al. 2011; Jha et al. 2007). Considering that *Xoo* mainly lives inside xylem vessels, the major building blocks of which are xylan and cellulose, it is no surprise that xylanases, lipases and cellulases are key virulence factors of *Xoo*. Moreover, genetic and phenotypic analyses have revealed extensive functional redundancy among these enzymes (Jha et al. 2007; Rajeshwari et al. 2005). Although *Xoo* is also equipped with several genes encoding proteases and pectinases, their function and importance in the pathogenicity of *Xoo* remain largely unclear except for one report by Xu and Gonzalez (1989) showing that a protease-deficient *Xoo* mutant exhibits impaired growth and reduced virulence in susceptible rice varieties (Lee et al. 2005; Wang et al. 2008; Furutani et al. 2004; Xu and Gonzalez 1989).

Table 2.1 List of extracellular enzyme encoding genes that are functionally characterized in *Xoo*

Gene	Protein	Function	Reference
bglXoB	Beta-glucosidase	Important	Temuujin et al, 2011;
eglXoA	Endoglucanase	Vital	Temuujin et al, 2011;
eglXoB	Endoglucanase	Vital	Temuujin et al, 2011; Hu et al, 2007.
celbXoA	1,4-Beta-cellobiosidase	Important	Temuujin et al, 2011; Jha et al, 2007.
celXoB	Cellulase	Vital	Temuujin et al, 2011; Jha et al, 2007.
bglXoF	Beta-glucosidase	Important	Temuujin et al, 2011;
bglXoA	Cellulase S	NI	Temuujin et al, 2011;
bglXoC	Beta-glucosidase	NI	Temuujin et al, 2011;
bglXoD	1,4-Beta-glucosidase	NI	Temuujin et al, 2011;
eglXoC	Endoglucanase	NI	Temuujin et al, 2011;
bglXoE	Cellulase	NI	Temuujin et al, 2011;
bglXoG	1,4-Beta-glucosidase	NI	Temuujin et al, 2011;

xynA	Xylanase	NI	Temuujin et al, 2011;
xynB	Xylanase	Vital	Rajeshwari et al, 2005;
lipA	Lipase/esterase	Vital	Rajeshwari et al, 2005; Aparna et al, 2009.
cysP2	Cysteine protease	ND	Furutani et al, 2003
ecpA	Extracellular protease	NI	Zou et al, 2012

Vital: absolutely required for *Xoo* pathogenicity; Important: important but not vital; NI: not important; ND: not determined.

Extracellular wall degradative enzymes are usually secreted by the type II secretion system (T2SS). The T2SS apparatus consists of 12-16 components, designated as protein A to S, most of which are associated with the bacterial inner membrane (Jha et al. 2005). Recent sequence analysis of *Xanthomonas* spp. revealed two gene clusters, designated *xcs* and *xps*, encoding two T2SSs (Büttner and Bonas 2010). The first identified T2SS (Xcp) is involved in secretion of various proteins, while the second one (Hxc, homologous to Xcp) is involved in secretion of an alkaline phosphatase (LAP) (Jha et al. 2005; Korotkov et al. 2013). Contrary to other *Xanthomonas* species, *Xoo* is only equipped with the first one (Büttner and Bonas 2010). Within this T2SS, protein D acts as a secretin that forms oligomeric rings in the outer bacterial membrane, while proteins E and F are indispensable components of an inner membrane complex which serves as a platform for the assembly of pseudopili (Filloux 2004). Mutations of *xpsD*, *xpsE* and *xpsF*, lead to impaired secretion of xylanase, lipases and cellulases, as well as reduced virulence (Ray et al. 2000; Furutani et al. 2004; Sun et al. 2005).

In addition to their role in bacterial pathogenesis, cell wall-degrading enzymes also act as elicitors of plant immune responses (Ryan and Farmer 1991). Infiltration of rice leaves with purified cellulase, cellobiosidase, or lipase, triggers enhanced accumulation of callose deposits, cellular browning and increased lignification, as well as resistance to subsequent *Xoo* infection (Jha et al. 2007). The elicitor activity of these enzymes is believed to be due to the release of oligo-galacturonides and other so-called damage-associated molecular patterns (DAMPs), which can be defined as endogenous signals originating from stressed or injured cells.

2.3.2.4 Effectors and the type III secretion system

During the evolutionary arms race between plants and their attackers, successful pathogens have evolved the ability to intercept PAMP-induced responses by injecting into host cells a suite of effector proteins that collectively contribute to the pathogen's virulence. Although there are exceptions, most effector proteins are secreted by the type III secretion system (T3SS) encoded by the *hrp* (hypersensitive reaction and pathogenicity) gene cluster. In *Xoo*, the AraC-type transcriptional activator HrpX plays an important regulatory role in modulating expression of *hrp* genes (*hrpB* to *hrpF*) that are responsible for assembly of the T3SS apparatus, as well as genes encoding T3SS effectors. Accordingly, *hrp* null mutants display defects in effector secretion and an almost complete loss of pathogenicity (Tsuge et al. 2014).

Based on protein structure and their mode of action, T3SS effectors can be broadly categorized into two groups: TAL (transcription activator-like) and non-TAL effectors. TAL effectors (TALE), also known as AvrBs/PthA effectors, mimic eukaryotic transcription factors and directly induce expression of plant genes by binding to cognate promoter boxes. TALs possess several characteristic structural features, including a central region of near-perfect 34-amino-acid repeats which mediate protein dimerization and DNA binding. This central repetitive region determines the sequence specificity of the cognate target gene as two hypervariable amino acid residues at position 12 and 13 in each repeat recognize one base pair in the target DNA. In addition to the central repeat region, TALEs also contain nuclear localization signals and an acidic transcription activator-like domain in the C terminus which are essential for protein function and mediate nuclear import and activation of plant gene expression, respectively (Leach and White 1996).

Over the past few years, genome mining approaches have led to the identification of at least 19 *Xoo* genes predicted to encode TAL effectors (Table 2.2) (Mondal et al. 2014). Among these, PthXo1, PthXo2 and PthXo3 are three of the best-studied TALs , and the cognate rice genes function as major susceptibility genes (White and Yang 2009). PthXo1 activates the expression of *Xa13*, (Yang et al. 2006) also named *Os8N3* and *OsSWEET11*, a dominant rice gene encoding a MtN3-family membrane protein that in healthy rice plants is involved in pollen development (Yang et al. 2006; Chu et al. 2006). Activation of *Os8N3* is important for *Xoo* growth and disease development in rice. The *Os8N3* RNAi plants and plants harboring the allelic recessive R gene *xa13* are resistant to *Xoo* expressing PthXo1. Promoter sequence analysis revealed that *xa13*-mediated resistance is due to mutation of the UPT_{PthXo1} box in the *xa13* promoter, which results in PthXo1 carrying strains being unable to induce *xa13* expression. Interestingly, this resistance can be overcome by another TAL effector, AvrXa7, that does not induce *Os8N3*, but another member of the rice MtN3 family,

Os11N3 (also named *OsSWEET14*) (Chen et al. 2010; Antony et al. 2010). Interestingly, the Os11N3 and Os8N3 proteins function as low-affinity glucose transporters in animal cell lines, suggesting that they may supply sugars to *Xoo* by an efflux mechanism (Chen et al. 2010). In addition, Os8N3 interacts with two plasma membrane-localized copper transporter-type proteins, COPT1 and COPT5, to promote removal of copper from xylem vessels (Yuan et al. 2010). Copper inhibits *Xoo* growth and strains expressing PthXo1 are more sensitive to copper than others. Therefore, it seems that PthXo1 helps *Xoo* to overcome basal rice defenses by promoting the redistribution of copper (Yuan et al. 2010). Much like PthXo1-induced susceptibility, PthXo2 defeats rice immune responses by transcriptionally activating the dominant susceptibility gene *Xa25* through binding to the cognate UPT_{PthXo2} box. However, contrary to Xa13, Xa25 does not interact with COPT1 and COPT5, suggesting a different mode of action (Liu et al. 2011).

Other important *Xoo* TALEs are AvrXa10 and AvrXa27, both of which are recognized in resistant rice plants by the dominant resistance genes *Xa10* and *Xa27*, resulting in HR elicitation and inhibition of bacterial growth (Gu et al. 2009; Tian et al. 2014). In contrast, resistance conferred by the recessive *R* gene *xa5* is based on a mutated gamma subunit of the general transcription factor IIA 5 (TFIIAγ5) (Jiang et al. 2006; Iyer and McCouch 2004). It is hypothesized that TFIIAγ encoded by the dominant susceptibility gene *Xa5* cooperates with *Xoo* TALEs to induce the expression of susceptibility genes required for *Xoo* invasion, whereas the mutated TFIIAγ5 encoded by *xa5* would induce passive resistance by attenuating TALE-activated host gene expression (Iyer-Pascuzzi et al. 2008).

Compared to the relative wealth of knowledge on TAL effectors, little is known about the roles of *Xoo* non-TAL effectors, also referred to as Xanthomonas outer proteins (Xop). Thus far, 22 open reading frames have been predicted to encode Xop proteins (Mondal et al. 2014). Although lacking widely conserved structures, Xops share some common features in the N-terminal amino acid composition, and most of them possess at least three of the following characteristics: 1) more than 20% Ser and Pro residues; 2) less than 6% Leu residues in the first 50 amino acid residues; 3) either 0 or 1 acidic amino acid residue (Asp or Glu); and 4) Leu, Ile, or Pro at the third or fourth residue (Furutani et al. 2009). Among the predicted Xops, XopZ was reported to contribute to PXO99 pathogenicity by interfering with cell-wall-related PTI (Song and Yang 2010). XopR, likewise, has been shown to inhibit PTI responses such as callose deposition, and is important for full virulence of *Xoo* (Akimoto-Tomiyama et al. 2012; Zhao et al. 2013). In addition, XopN, one of the most important Xops in *Xoo* strain KXO85, was found to interact with OsVOZ2 and OsXNP in rice (Cheong et al. 2013). While the function of OsVOZ2 remains unclear, OsXNP has recently been identified

as a putative thiamine synthase. Thiamine is known to act as a systemic defense activator in plants (Ahn et al. 2005). Therefore, by interacting with OsXNP, XopN may disrupt thiamine synthesis and thereby attenuate the defense response of rice (Cheong et al. 2013). These studies all point out that Xops function as virulence factors by modulating plant defense signaling.

Table 2.2 Distribution of genes encoding TAL- and non-TAL-effectors in three *Xoo* strains (Mondal et al. 2014)**.**

	PXO99A	KACC10331	MAFF311018
Xop effectors			
xopAA	PXO_00234	XOO3022	XOO_2875
xopAB	PXO_01867	XOO3338	XOO_3150
xopAC	-	-	-
xopAD	PXO_03833	XOO4401	XOO_4145
xopAE	PXO_03420	XOO0065	XOO_0110
xopAF	-	-	-
xopAG	-	-	-
xopAH	?	?	?
AvrBS1	-	-	-
AvrBS2	PXO_03330	XOO0168	XOO_0148
xopB	-	-	-
xopC	PXO_02108	XOO3424	XOO_3221
xopD	-	-	-
xopE	-	-	-
xopF1	PXO_03413	XOO0074	XOO_0103
xopG	?	XOO4523	XOO_4258
xopH	-	-	-
xopK	PXO_01625	XOO1768	XOO_1169
xopL	PXO_01620	XOO1762	XOO_1162
xopN	PXO_02760	XOO0343	XOO_0315
xopO	-	-	-
xopP	PXO_02107	XOO3426/XOO3425	XOO_3222

xopQ	PXO_03901	XOO4466	XOO_4208
xopR	PXO_03819	XOO4391	XOO_4134
xopT	-	XOO4824	XOO_2210
xopU	PXO_00236	?	XOO_2877
xopV	PXO_04172	XOO4033	XOO_3803
xopW	PXO_03356	XOO0134	XOO_0037
xopX	PXO_03702	XOO4287	XOO_4042
xopY	PXO_04865	XOO1604	XOO_1488
xopZ1	PXO_06125/1041	XOO2543	XOO_2402
xopZ2	-	-	-
TAL effectors			
tal1/pthXo7	PXO_03922	XOO1237	XOO_1232
tal2a	PXO_00223	XOO3013	XOO_2865
tal2b/pthXo1	PXO_00227	XOO2131	XOO_4255
tal3a	PXO_00511	XOO3014	XOO_2866
tal3b	PXO_00505	XOO2275	XOO_2667
tal4	PXO_00318	XOO2128	XOO_2129
tal5a	PXO_00567	?	XOO_2864
tal5b/pthXo6	PXO_00572	XOO2279	XOO_2160
tal6a	PXO_00546	?	XOO_2158
tal6b	PXO_05609	XOO2276	XOO_2127
tal7a	PXO_05633	?	?
tal7b	PXO_01085	XOO2276	?
tal8a	PXO_06229	?	?
tal8b	PXO_06234	?	?
tal9a	PXO_02172	?	?
tal9b	PXO_05714	?	?
tal9c/avrXa27	PXO_05718	XOO2276	XOO_1134
tal9d	PXO_02269	?	?
tal9e	PXO_02272	?	XOO_2001

-: not present; ?: present as homologs or pseudogenes.

Interestingly, accumulating evidence indicates that the T3SS closely interacts with and promotes T2SS to further enhance *Xoo* virulence. Indeed, expression of some genes encoding type II secretory proteins relies on the T3SS, while T3S effectors often suppress innate rice defense responses that are induced by T2SS effectors. (Jha et al. 2007; Furutani et al. 2004).

2.3.2.5 Xanthomonadins

One of the most salient features of *Xanthomonas* spp. is their ability to produce a unique group of halogenated, aryl-polyene yellow pigments, called xanthomonadins (Kennedy and En 1977). Like EPS, xanthomonadins serve multiple functions, protecting bacteria from photobiological and peroxidative damage, as well as promoting epiphytic survival (Poplawsky et al. 2000; He et al. 2011). Accordingly, xanthomonadin-deficient *Xoo* mutants display strongly reduced virulence compared to wild-type bacteria (Goel et al. 2001). The *pig* gene cluster, involved in a type II polyketide synthase pathway, has been reported to be responsible for the biosynthesis and outer membrane localization of xanthomonadins (Goel et al. 2002). Initially, xanthomonadin production was believed to be mediated by a diffusible factor (DF), which was later identified as 3-hydroxybenzoic acid (3-HBA) (Poplawsky and Chun 1997; He et al. 2011). Goel et al. (2002) demonstrated that xanthomonadin synthesis in *Xoo* strain BXO1 is encoded by a 21 kb gene cluster that contains seven transcriptional units, designated *pigA* to *pigG* (Goel et al. 2002). Analysis of the *pigB* DNA sequence revealed the presence of two open reading frames, the second one (*xanB2*) being responsible for synthesis of DF (Poplawsky and Chun 1997; He et al. 2011). Recent work by Zhou et al. (2013a,b), however, uncovered XanB2 as a bifunctional chorismatase that hydrolyzes chorismate to produce both DF (3-HBA) and 4-HBA (Zhou et al. 2013b, 2013a). Null mutations in *xanB2* lead to xanthomonadin deficiency, lower resistance to H_2O_2 and impaired virulence of *Xoo* (Zhou et al. 2013a). Similarly, the *aroE* mutant of *Xoo*, which is deficient in converting shikimate to chorismate, was unable to produce xanthomonadins and is much less virulent than the WT strain (Goel et al. 2001). Taken together, these findings clearly demonstrate the importance of xanthomonadins in maintaining full virulence of *Xoo*.

2.3.2.6 Cell to cell communication mechanisms

Although bacteria exist as individual cells, they can also act collectively as multicellular organisms via sophisticated cell-to-cell communication systems. This phenomenon, known

as quorum sensing (QS), enables bacteria to track changes in cell density and adjust gene expression accordingly. QS has long been found to play a vital role in bacterial pathogenicity by coordinating the expression of multiple virulence traits through the production, detection, and response to extracellular signal molecules called autoinducers (AI). At low population density, bacteria produce basal levels of AI signals, which diffuse into the extracellular space, thus preventing detection by bacterial receptors. At high density, however, accumulated AI signals reach a certain threshold level, thereby setting off a wide variety of pathogenicity-related processes. As such, QS enables bacteria to quickly adapt to environmental conditions and invest in energy-consuming virulence traits only when the impact of these processes on the host will be maximized.

Like other Xanthomonads, *Xoo* deploys the diffusible signal factor (DSF)-mediated QS system to coordinate several virulence traits including production of EPS and extracellular enzymes, biofilm formation and motility (He et al. 2010; Rai et al. 2012; Chatterjee and Sonti 2002; Jeong et al. 2008; Deng et al. 2005; Ryan and Dow 2011) (Figure 2.5). To date, 13 DSF-family signals have been identified, all of which are unsaturated fatty acid-like molecules (Deng et al. 2005). Among them, DSF, BDSF and CDSF (Figure 2.4) are produced by *Xoo*, DSF being the most abundant and most bioactive (He et al. 2010). Production of DSF depends on the *RpfB* and *RpfF* genes, encoding a putative long chain fatty acyl CoA ligase and a putative enoyl-coA hydratase, respectively (Barber et al. 1997; Zhao et al. 2011). Downstream of DSF biosynthesis, perception and transduction of DSF signals occurs through a conserved phosphorelay mechanism governed by the RpfC/RpfG two-component system (Ryan et al. 2006; He et al. 2006; Slater et al. 2000). Interestingly, RpfC not only senses DSF through an unknown mechanism, but also negatively regulates DSF biosynthesis via direct protein-protein interactions (Cheng et al. 2010). RpfG, on the other hand, transmits DSF signals by influencing the level of the secondary QS messenger cylic-di-GMP either by activating a set of GGDEF- or EAL-domain proteins, or by directly degrading cylic-di-GMP via its HD-GYP domain (Ryan et al. 2010; von Bodman et al. 2008). GGDEF-domain-containing proteins generally function as diguanylate cyclases that synthesize cyclic-di-GMP, whereas the HD-GYP and EAL domain proteins act as phosphodiesterases that degrade c-di-GMP. Cylic-di-GMP and its receptor, CAP-like protein (CLP), are global regulators that control various virulence-associated processes, and are widely conserved in a range of bacterial species (Simm et al. 2004; He et al. 2007; Ryan 2013). Accordingly, recent findings showed that in *Xoo*, turnover of cylic-di-GMP is correlated with the pathogen's virulence (Yang et al. 2012). Similarly, deletion of the CLP-encoding gene in *Xoo* strain KACC 10859 attenuated virulence (Cho et al. 2011). Together with the

loss-of-virulence phenotype caused by null mutations in *RpfB, RpfF, RpfC* or *RpfG*, these findings underscore the central importance of DSF signaling in *Xoo* virulence.

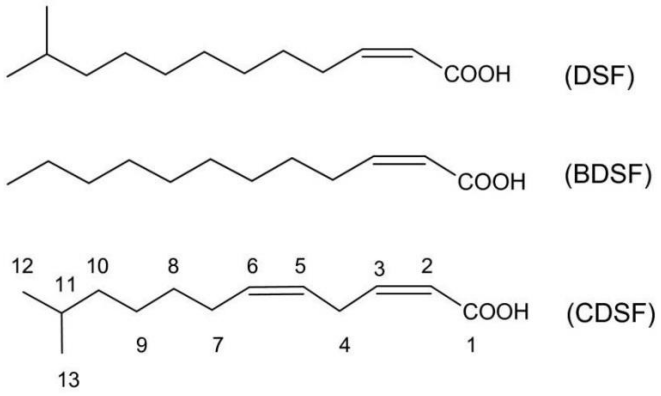

Figure 2.4 Chemical structures of DSF-type molecules produced by *Xoo* (He et al, 2010).

In addition to DSF-governed QS, there is evidence for a second QS circuit in *Xoo* that is mediated by a Diffusible Factor (DF), characterized as 3-hydrobenzoic acid. As mentioned before, this DF QS system is mainly responsible for regulation of xanthomonadin production (Zhou et al., 2013). It has been shown that *XanB2* catalyzes synthesis of both 3-hydrobenzoic acid (3-HBA) (DF) and 4-HBA, which is mainly used as a precursor for coenzyme Q (CoQ) biosynthesis (Zhou et al. 2013b). Acting as a vital cofactor in the aerobic respiratory electron transport chain, CoQ also serves as an antioxidant that protects membrane phospholipids and proteins from lipid peroxidation (Cluis et al. 2007). Interestingly, *xanB2* deletion mutants exhibit a pleiotrophic phenotype, including xanthomonadin deficiency, impaired EPS production, reduced viability and H_2O_2 tolerance, and lower virulence (Zhou et al. 2013b). Many of these traits are also controlled by the DSF-mediated circuit, suggesting possible interplay and functional redundancy among both QS systems (Zhou et al. 2013b). It has been proposed that utilization of different mechanisms to modulate similar virulence traits may provide *Xoo* with plasticity in response to various environmental cues (He et al. 2011).

Canonical QS systems consist of a LuxI-family synthase responsible for synthesizing N-acyl homoserine lactone (AHL) signals and a cognate LuxR-family transcriptional regulator that binds AHLs at quorum concentrations. Despite not producing any AHL-like molecules and lacking an intact LuxI/LuxR system, *Xoo* does possess a protein having the same modular structure of LuxR proteins, designated OryR (Ferluga et al. 2007). Since there is not a cognate LuxI-family synthase gene in the genome, OryR can be considered an unpaired or orphan LuxR-type response regulator. Lacking cognate LuxI proteins, LuxR solos are able to

regulate target genes by either responding to endogenous AHLs, by sensing AHLs produced by neighboring bacteria, or by interacting with eukaryotic signals. As such, LuxR-like solos seem to function as master messengers involved in both interspecies and interkingdom communication (Gonzalez and Venturi 2013; Subramoni and Venturi 2009). Biochemical studies by the Venturi lab revealed that OryR does not respond to AHLs, but interacts with an unknown rice signal (RSM) to activate downstream virulence genes (Ferluga and Venturi 2009). Moreover, genome-wide transcriptome analysis revealed that approximately 8% of all ORFs in the *Xoo* genome are affected by OryR mutation, suggesting a widespread role of OryR in orchestrating rice-*Xoo* interkingdom signaling (Gonzalez et al. 2013).

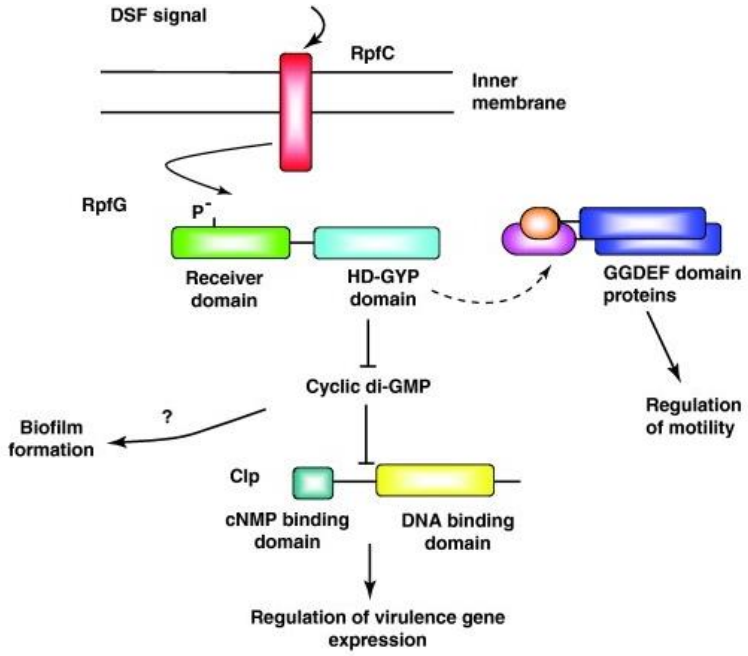

Figure 2.5 Model depicting DSF signaling pathway in *Xanthomonas* (Ryan and Dow 2011)**.**

2.3.2.7 Manipulating plant hormones pathways

Plant hormones are key regulators of the plant's defense signaling network, controlling a wide variety of constitutive and inducible defense responses. It is therefore not surprising that many pathogens have evolved mechanisms to tap into the plant hormone signaling network in order to interfere with host resistance. In general, plant pathogens appear to manipulate the hormone signaling circuitry of plant hosts by one of three basic strategies: 1) production of plant hormones or mimics thereof; 2) perturbation of plant hormone production; 3) modulation of hormone signaling pathways (Melotto and Kunkel 2013).

Despite its widespread distribution and economic importance, our understanding of the hormone pathways controlling disease and resistance against *Xoo* is still in its infancy. Nevertheless, accumulating evidence suggests that SA, JA and brassinosteroid (BR) are positive regulators of resistance against *Xoo*, whereas ABA, auxin, gibberellins (GAs), CK and ET appear to promote disease susceptibility (De Vleesschauwer et al. 2013). Interestingly, virulent *Xoo* strains have been found to secrete the main auxin indole-3-acetic acid (IAA) and it is hypothesized that this *Xoo*-derived IAA may in turn induce rice to synthesize its own IAA at the infection site (Fu et al. 2011). At the same time, *Xoo* also harbors the key CK biosynthesis enzyme isopentenyl transferase, suggesting that the bacterium may also be able to synthesize CK (Salzberg et al. 2008). Besides producing plant hormones itself, *Xoo* also seems to actively interfere with hormone biosynthesis and signaling processes in infected rice leaves. For instance, susceptible rice varieties responding to *Xoo* infection are characterized by strong increases in ABA content and extensive transcriptional reprogramming of ABA-biosynthesis and ABA-responsive genes (Chapter 4). Furthermore, *Xoo* also seems to manipulate the rice GA pathway as both GA biosynthesis and catabolism genes were found to be strongly deregulated following infection (Yang et al. 2008). Although preliminary, these findings strongly suggest that *Xoo* targets multiple rice hormone pathways in order to disturb host hormone homeostasis and promote bacterial pathogenesis.

Chapter 3

Making sense of hormone-mediated defense networking: from rice to Arabidopsis

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Abstract

Plant hormones are small molecules that play diverse roles throughout the lifespan of plants. They orchestrate intrinsic developmental programs, convey environmental cues and drive adaptive responses to abiotic and biotic stresses. Historically, research aimed at elucidating the role of hormones in plant innate immunity has tended to focus on the use of experimentally tractable dicot plants such as *Arabidopsis thaliana*. Emerging from these studies is a picture whereby complex crosstalk and induced hormonal changes mold plant health and disease, with outcomes largely dependent on the lifestyle and infection strategy of invading pathogens. However, recent studies in monocot plants are starting to provide additional important insights into the immune-regulatory roles of hormones, often revealing unique complexities. In this review, we address the latest discoveries dealing with hormone-mediated immunity in rice, one of the most important food crops and an excellent model for molecular genetic studies in monocots. Moreover, we highlight interactions between hormone signaling, rice defense and pathogen virulence, and discuss the differences and similarities with findings in Arabidopsis. Finally, we present a model for hormone defense networking in rice and outline avenues for further research.

Introduction

Plant hormones are small signaling molecules that play pivotal roles in the regulation of plant growth, development, reproduction and survival. They not only orchestrate intrinsic developmental programs but also convey environmental inputs and drive adaptive responses to a wide variety of biotic and abiotic stresses. Plants typically respond to pathogen infection or herbivore attack with a complex scenario of sequential, antagonistic or synergistic action of different hormone signals leading to defense gene expression (Robert-Seilaniantz et al. 2011). This interplay or so-called crosstalk among individual hormone pathways enables plants to adjust their inducible defense arsenal to the type of attacker encountered and to use their limited resources in a cost-efficient manner (Pieterse et al. 2012, 2009).

Historically, plant hormone research has been polarized towards the use of the experimentally tractable dicot plant *Arabidopsis thaliana*. In this model species, the production and joint role of salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) upon pathogen attack is well studied and these three hormones are considered to be the key players in the regulation of the disease signaling pathways. Following microbe perception, plants produce a complex blend of SA, JA and ET, with the exact combination seemingly

depending on the infection strategy and lifestyle of the invading pathogen. Although there are exceptions, SA is usually effective against biotrophic pathogens that feed on living plant tissues, whereas cell death-provoking necrotrophic pathogens are commonly deterred by JA- and ET-dependent defenses (Bari and Jones 2009). Moreover, these two pathways most often interact in an antagonistic manner, which has led many authors to suggest that plant immunity follows a binary model with SA and JA/ET having opposite roles. In compliance with this concept, many other hormones, including abscisic acid (ABA), gibberellins (GA), auxins and cytokinins (CK), have been shown to differentially affect Arabidopsis resistance against biotrophs and necrotrophs by feeding into the SA-JA-ET cascades (Robert-Seilaniantz et al. 2011, 2007).

Although Arabidopsis has been an excellent model for studying hormone defense networking, recent studies using alternative model systems such as rice (*Oryza sativa L*.) are starting to provide important new insights, often revealing unique complexities (Sharma et al., 2013). Consumed daily by more than 3 billion people worldwide and accounting for up to 50% of the daily caloric uptake of the world's poor, rice is arguably the world's most important staple food. Moreover, due to its relatively small and fully sequenced genome, its ease of transformation, accumulated wealth of genetic and molecular resources, and extensive synteny and collinearity with other cereals, rice has emerged as an excellent model for molecular genetic studies in monocots (Jung et al., 2008).

Here, we survey recent advances dealing with hormone-regulated defense networking in rice, focusing on interactions between hormone signaling, rice defense and pathogen virulence. We pay special attention to the differences and similarities with findings in Arabidopsis and propose a model for defense signaling in rice that challenges the commonly accepted dichotomy between the effectiveness of hormone pathways and the lifestyle of a given pathway. For more detailed information on innate immune mechanisms and hormone biology in rice and other cereals, we refer the reader to a number of excellent recent reviews.

Salicylic acid: it's not all about NPR1

Salicylic acid is a natural phenolic compound that plays well-known roles in the regulation of a wide variety of immune responses triggered by pathogen-associated molecular patterns (PAMPs) and microbe-secreted effector proteins (Vlot et al. 2009). Following pathogen infection, endogenous levels of SA and its conjugates increase dramatically, immediately preceding the induction of pathogenesis-related proteins and the onset of local and systemic acquired resistance (SAR) (Malamy et al., 1990; Metraux et al., 1990).

In rice, however, the role of SA in the immune signaling network is still poorly understood, and even controversial. Driving the debate initially was the observation that rice accumulates high basal levels of SA (8 to 37 μg g^{-1} fresh weight) that do not change significantly upon pathogen attack (Silverman et al. 1995). In contrast, in tobacco and Arabidopsis, basal levels of SA are low (less than 100 ng g^{-1} fresh weight), but increase by two orders of magnitude following infection (Malamy and Klessig 1992). Moreover, unlike dicots where *de novo* synthesized SA is rapidly converted into SA β-glucoside, in rice most SA is present in the free acid form (Silverman et al. 1995). Interestingly, these high levels of free SA are hypothesized to function as a preformed antioxidant, protecting rice plants from oxidative damage caused by aging, pathogen attack or abiotic stress (Yang et al. 2004). However, considering the well-established role of SA as an inducer of biotroph resistance in dicots, it is tempting to speculate that these constitutively high SA levels may also explain why there are no rice pathogens with an exclusively biotrophic lifestyle such as rusts or powdery mildews.

Despite its high endogenous SA content, rice is not insensitive to exogenously administered SA, but this is plant age-dependent. For instance, topical application of SA triggers resistance to the hemibiotrophic blast fungus *Magnaporthe oryzae* in adult plants but not in young seedlings (Iwai et al. 2007). Moreover, synthetic SA analogs such as probenazole, benzothiadiazole (BTH) and tiadinil induce defense responses in rice and can enhance resistance to a wide range of pathogens with different lifestyles and infection strategies. Considering that tiadinil and BTH act downstream of SA biosynthesis and that SA-deficient rice plants expressing the bacterial salicylate hydroxylase *NahG* display unaltered *PR* gene expression (Xiong and Yang 2003), these findings suggest that the signaling action of SA, rather than its *de novo* biosynthesis, is an important factor mediating defense mobilization in rice.

Interestingly, the SA signaling pathway in rice shares several downstream components with the SAR pathway in Arabidopsis, including the master regulatory protein NPR1. During SAR, SA-induced redox changes reduce the intermolecular disulphide bonds that normally keep NPR1 in an inactive oligomeric state in the cytosol. This reduction in turn releases monomeric NPR1, which is translocated to the nucleus where it interacts with TGA transcription factors to activate defense gene expression. Recently, it was reported that NPR1 as well as its paralogs NPR3 and NPR4 also serve as SA receptor proteins (Wu et al. 2012; Fu et al. 2012).

To date, five NPR1-like genes have been identified in the rice genome, among which OsNPR1 (also called OsNH1) is the closest homolog of AtNPR1 (Yuan et al. 2007). Ectopic expression of *OsNPR1* in rice induced constitutive accumulation of *PR* transcripts, conferring

high levels of resistance to *Mo* and the hemibiotrophic leaf blight pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (Chern et al. 2005; Yuan et al. 2007; Sugano et al. 2010). By contrast, in rice and Arabidopsis overexpressing *AtNPR1*, defense-related genes are not activated until induced by abiotic stress, pathogen attack or BTH treatment (Cao et al. 1998; Fitzgerald et al. 2004).

Moreover, unlike the situation in Arabidopsis where nearly 99% of all BTH-responsive genes are controlled by AtNPR1, the rice SA circuit appears to branch into two distinct pathways mediated by OsNPR1 and the transcription factor OsWRKY45, respectively (Shimono et al. 2007; Takatsuji et al. 2010). OsWRKY45 was originally identified as a BTH-responsive TF that is essential for *R* gene- and plant activator-mediated resistance to *Xoo* and *Mo* (Shimono et al. 2007; Inoue et al. 2013; Shimono et al. 2012). Recently, it was reported that OsWRKY45 undergoes continuous degradation by the ubiquitine-proteasome system (UPS) in the nucleus, preventing spurious activation of defense responses in the absence of pathogen attack (Matsushita et al. 2013). In addition, UPS-mediated OsWRKY45 turnover is required for full-scale activation of the transcriptional activity of the protein following SA treatment or pathogen attack. Interestingly, in Arabidopsis, AtNPR1 is regulated in a similar way, whereas proteasome degradation of OsNPR1 in rice has not been observed yet (Spoel et al. 2009; Matsushita et al. 2013).

Together, these results highlight the unique complexities associated with SA signal transduction in rice. Most notably, the rice SA pathway seems to branch into two sub-pathways controlled by OsNPR1 and OsWRKY45. Intriguingly, recent microarray experiments revealed that almost half of all BTH-responsive genes and over two thirds of all BTH-downregulated genes are dependent on OsNPR1. These downregulated genes include many genes involved in photosynthesis and protein synthesis, suggesting a novel function of OsNPR1 in relocating energy and resources from house-keeping cellular activities to defense reactions (Sugano et al. 2010). In contrast, most genes upregulated by BTH are dependent on OsWRKY45, including many *PR* marker genes (Takatsuji et al. 2010; Matsushita et al. 2013). Together these findings favor a scenario whereby OsNPR1 and OsWRKY45 play different yet complementary roles in the rice SA pathway with OsNPR1 acting as an energy switch, enabling limited resources to be diverted to OsWRKY45-mediated pathogen defense.

Jasmonic acid

Jasmonic acid and its derivatives, collectively known as jasmonates, are lipid-derived hormones that regulate numerous physiological processes, including wound responses,

secondary metabolite synthesis, and defense against biotic and abiotic stresses (Dombrecht et al. 2007). In dicots, JA is widely believed to be predominantly effective against necrotrophic pathogens and herbivorous insects, whereas SA signaling is typically associated with immunity against biotrophs (Glazebrook 2005). Moreover, although there is evidence for both positive and negative relationships between both pathways, the primary mode of interaction appears to be mutual antagonism, with corresponding trade-offs between biotrophs, on the one hand, and resistance to necrotrophs, on the other (Bostock 2005). This SA-JA antagonism is evolutionary widely conserved and has been reported in as many as 17 plant species in various taxonomic groups (Thaler et al. 2012).

In rice, however, strikingly different results have been obtained with reports implicating JA in resistance against pathogens with distinct lifestyles and infection strategies. Perhaps most intriguingly, studies with JA-modified transgenics and pharmacological inhibitor experiments have uncovered JA as a powerful activator of resistance against the (hemi)biotrophic pathogens *Xoo* and *M. oryzae* (Mei et al. 2006; Yamada et al. 2012; Riemann et al. 2013; Taniguchi et al. 2013). However, application of JA also conditions immunity against the necrotrophic sheath blight fungus *Rhizoctonia solani* (Taheri and Höfte 2007). Enhanced sheath blight resistance concomitant with increased JA levels and induction of JA-responsive gene expression was also observed in rice plants overexpressing the pathogen-inducible transcription factor gene *OsWRKY30* (Peng et al. 2012). In addition, the JA pathway has been repeatedly implicated in rice defenses against root-knot nematodes and insect herbivores (Nahar et al. 2012; Zhou et al. 2009; Ye et al. 2012). Together these findings challenge the common assumption that JA triggers resistance to necrotrophs and susceptibility to biotrophs, and suggest that in rice there is no dichotomy between the effectiveness of the JA pathway and the lifestyle of the invading pathogen.

Despite the ability of JA to induce resistance against both biotrophic and necrotrophic rice pathogens, several reports indicate that SA-JA antagonism is conserved in rice. In roots, SA attenuated JA-induced expression of the rice *PR* gene *RSOsPR10* and its negative regulator *OsERF1* (Takeuchi et al. 2011). Furthermore, in wounded rice plants, JA levels rise whereas SA levels decrease, suggesting negative crosstalk in the direction of JA damping SA action (Lee et al. 2004). Further evidence supporting antagonistic SA-JA signal interactions comes from gene expression experiments demonstrating enhanced transcript accumulation of the JA-responsive genes *OsAOS2* and *JaMYB* in SA-deficient *NahG* rice (Lee et al. 2001; Mei et al. 2006).

Over the past few years, various regulatory components involved in SA-JA crosstalk have been identified, key among which is NPR1 (Spoel et al. 2003; Koornneef and Pieterse 2008;

Thaler et al. 2012). Like its Arabidopsis counterpart, overexpression of *OsNPR1* is characterized by strong activation of SA-responsive genes and concomitant suppression of JA marker genes (Yuan et al. 2007). Similar to the situation in dicots, nuclear localization of OsNPR1 is required for SA-mediated defense gene expression, but not for suppression of JA signaling (Yuan et al. 2007; Spoel et al. 2003). *OsNPR1* antisense plants display elevated levels of JA and increased expression of JA biosynthetic genes upon insect infestation (Li et al. 2013). Accordingly, ectopic expression of *OsNPR1* not only confers enhanced resistance to *M. oryzae* and *Xoo*, but also renders plants more susceptible to herbivorous insects. In a similar vein, rice plants overexpressing AtNPR1 are hypersensitive to light (Fitzgerald et al. 2004) and display an increased susceptibility to viral infection and reduced tolerance to abiotic stress (Quilis et al. 2008). Together, these findings suggest that OsNPR1 acts as a positive regulator of SA-dependent pathogen resistance, while suppressing JA-mediated defenses to herbivorous insects and viral infection as well as tolerance to abiotic stresses. A role in SA-JA crosstalk has also been suggested for OsWRKY13. Functioning upstream of OsNPR1, this TF positively regulates SA-mediated rice defenses while suppressing the JA pathway (Qiu et al. 2008, 2007, 2009).

Although abovementioned studies clearly indicate the potential for negative SA-JA signal crosstalk, examples of positive interactions have also been reported, both in dicots and monocot systems. In general, however, positive SA-JA signal interactions appear to be more common in rice than in Arabidopsis. For instance, rice plants mutated in the hydroperoxide lyase *OsHPL3* display strongly enhanced JA levels concomitant with increases in SA production and heightened expression of SA-responsive *PR* genes (Liu et al. 2012b; Tong et al. 2012). Activation of JA synthesis was also found to prime herbivore-induced SA synthesis in rice plants silenced for the phospholipase D genes *OsPLDα3* and *OsPLDα4* (Qi et al. 2011). Moreover, microarray studies showed that more than half of all BTH- or SA-upregulated genes are also induced by JA (Garg et al. 2012; Tamaoki et al. 2013). Together, these findings bring a new twist to the classical crosstalk model and suggest that although hyperactivation of one has the ability to override the other, rice SA and JA pathways feed into a common rice defense system that is effective against different types of attackers. Although the underlying molecular mechanisms remain to be elucidated, recent findings by our lab suggest that such co-operative SA/JA defense signaling is orchestrated by the rice DELLA protein SLR1 (see below).

Ethylene

ET is a gaseous hormone that controls diverse aspects of plant life. In plant immunity, ET is generally thought to act in concert with JA to induce necrotroph resistance while antagonizing SA-mediated biotroph resistance (Derksen et al. 2013) . Accumulating evidence, however, indicates that ET can interact both positively and negatively with SA, depending on the infection strategy of the invading pathogen (Pieterse et al. 2012; Derksen et al. 2013). Like SA and JA, ET is rapidly synthesized following PAMP perception (Boller and Felix, 2009; Schwessinger and Ronald, 2012). Although the precise function of ET in PAMP-triggered immunity is still elusive, recent evidence suggest a combined role of ET and endogenous peptides in an amplification loop required for sustained PTI (Tintor et al., 2013; Liu et al., 2013).

In rice, accumulation of ET and its coproduct cyanide was found to be indispensable for effector-triggered immunity (ETI) against the hemibiotroph *Mo* (Iwai et al. 2007). Interestingly, activation of ET synthesis was also shown to be responsible for the partial blast resistance of rice plants growing in anaerobic conditions such as moisture-saturated soils or flooded paddies (Singh et al. 2004). Transgenic lines overexpressing *OsACS2*, a gene encoding the key ET biosynthesis enzyme 1-aminocyclopropane-1-carboxylic acid synthase (ACS), showed increased resistance to both *M. oryzae* and the necrotrophic fungus *R. solani*, whereas silencing of ET biosynthesis genes or the central ET signal transducer OsEIN2b rendered plants more susceptible against *M. oryzae* and the bacterial pathogen *Burkholderia glumae* (Bailey et al. 2009; Seo et al. 2011).

Abovementioned studies clearly indicate that ET plays an important role in rice defense to various pathogens. However, as in dicots, ET can also act negatively on rice immunity, as was shown for the necrotrophic rice brown spot fungus *Cochliobolus miyabeanus*. Exogenously applied Ethephon (which is converted to ET in plant cells) strongly promotes disease development in this interaction, whereas genetic or pharmacological disruption of ET signaling resulted in enhanced resistance (De Vleesschauwer et al. 2010). Moreover, gene expression experiments revealed a strong activation of ET signaling in susceptible but not in resistant rice plants. In conjunction with the ability of the fungus to produce ET itself (Van Bockhaven et al., unpublished), these findings strongly suggest that *C. miyabeanus* exploits ET as virulence factor and co-opts the rice ET signaling route to rewire the rice signaling circuitry and antagonize host immunity. A negative impact of ET on rice disease resistance has also been observed for *Xoo*. Shen et al. (2011) reported that silencing of the MAPKKK OsEDR1 resulted in reduced expression of *ACS* genes, low levels of ET, and enhanced resistance to *Xoo* (Shen et al. 2011). Interestingly, this resistance was accompanied with

increased SA and JA synthesis and constitutive expression of SA- and JA-marker genes, suggesting that when ET is lowered, levels of SA and JA increase (Shen et al. 2011). Together these observations suggest that ET plays a complex and ambiguous role in the rice immune system, the effect of which may depend not only on the lifestyle and overall infection biology of the attacking pathogen, but also on specialized features of each interaction.

Abscicic acid

Compared with the classic defensive hormones SA, JA and ET, the role of the 'abiotic stress hormone' abscisic acid (ABA) in regulating plant immunity is much less understood, and even controversial. Recent studies in dicots showed divergent and complex effects of ABA on defense responses, including the suppression of SA- and JA/ET-dependent defenses, synergistic crosstalk with JA signaling, suppression of ROS generation, induction of stomatal closure, and stimulation of callose deposition (Asselbergh et al. 2008; Cao et al. 2011). In general, the impact of ABA on plant defense seem to be plant-pathogen interaction-specific, rather than to rely on the lifestyle or infection strategy of the pathogen. In Arabidopsis, for instance, ABA both positively and negatively regulates resistance to the necrotrophic fungi *Alternaria brassicicola* and *Botrytis cinerea*, respectively (Adie et al. 2007). The timing of infection is another crucial factor underlying ABA modulation of plant immunity. This is nicely exemplified in *Pseudomonas syringae* pv. *tomato* (*Pst*)-infected Arabidopsis where ABA prevents pathogen entry by inducing stomatal closure, yet antagonizes post-invasive disease resistance by suppressing SA-depedendent defenses (Mohr and Cahill 2007; Melotto et al. 2006; Grant et al. 2007).

In common with these findings, ABA also plays ambiguous roles in the rice defense-signaling network. We previously showed that exogenous ABA enhances basal resistance of rice against the necrotrophic brown spot fungus *C. miyabeanus* (De Vleesschauwer et al. 2010). This ABA-inducible resistance was associated with restriction of fungal progression in the mesophyll and was dependent on negative crosstalk with the rice ET-signaling pathway (De Vleesschauwer et al. 2010). In a similar manner, it has been proposed that ABA conditions susceptibility to both *M. oryzae* and *Xoo* by antagonizing effectual SA-mediated defenses upstream or at the level of OsNPR1 and OsWRKY45 (Jiang et al., 2010; Xu et al., 2013). Interestingly, infection by *M. oryzae* and *Xoo* is tightly associated with greatly elevated ABA levels and extensive reprogramming of ABA-responsive genes (Ribot et al. 2008; Liu et al. 2012a; Xu et al. 2013). Consistent with previous findings in the Arabidopsis-*Pst* pathosystem (Grant et al. 2007; De Torres Zabala et al. 2009; Goel et al. 2008; Ho et al. 2013), it therefore

seems that both pathogens hijack the rice ABA pathway to cause disease. In support of this assumption, *M. oryzae* was recently shown to produce and secrete ABA *in vitro* and *in planta* (Jiang et al. 2010). Since ABA has no apparent impact on the pathogen's physiology, one may hypothesize that *M. oryzae* uses its own ABA to activate ABA signaling in host cells, thereby suppressing the SA- and ET-signaling pathways that normally serve to limit pathogen growth (Takatsuji and Jiang 2014).

Although understanding of molecular components governing signal transduction and sensitivity in the rice ABA signaling network is still in its infancy, accumulating evidence points towards a crucial role of the ABA-inducible protein kinase OsMPK5. *OsMPK5* RNAi lines show increased levels of ET and enhanced resistance to multiple hemibiotrophic pathogens including *M. oryzae*, *Xoo*, and *B. glumae* (Nahar et al., 2012; Xiong & Yang, 2003; Xu et al., 2013); however, they are also impaired in ABA-inducible resistance to *Cm* and are hypersensitive to abiotic stresses (Bailey et al. 2009; De Vleesschauwer et al. 2010). Conversely, silencing of the central ET-signal transducer *OsEIN2* resulted in enhanced resistance to *C. miyabeanus* as well as hypersensitivity to *M. oryzae*, *Xoo*, ABA and abiotic stress (Bailey et al. 2009; De Vleesschauwer et al. 2010). Together, these findings suggest that OsMPK5 and OsEIN2 act as molecular switches between the rice ABA and ET pathways, thereby differentially regulating abiotic stress tolerance and *C. miyabeanus* resistance, on the one hand, and defense against hemibiotrophic pathogens, on the other.

Interestingly, OsMPK5 positively interacts with the JA pathway in protecting rice against chewing herbivores, suggesting positive crosstalk in the direction of ABA boosting JA action (Wang et al. 2013). However, Nahar et al. (2013) reported that ABA disables JA-induced resistance against the migratory nematode *Hirschmaniella oryzae*, indicating that the nature of interaction between these pathways is complex and also attacker dependent.

New kids on the block: developmental hormones do defense

Contrary to the classic defense hormones SA, JA and ET, other hormones including auxins, GAs, BRs, and CKs, were historically best studied for their role in growth and development and only recently emerged as additional players in plant-microbe interactions. Although much remains to be discovered about their precise role and function in orchestrating plant defense, recent data are now beginning to unveil how these 'developmental' hormones modulate host immunity, and how microbe-induced perturbations of these classic growth regulators contribute to virulence.

Auxins

Auxins, such as indole-3-acetic acid (IAA), are a major class of plant hormones that control a range of cellular processes, including apical dominance, tropistic growth, lateral root formation, vascular tissue development, and regulation of plant senescence. Thus far, studies on Arabidopsis imply that auxin attenuates (hemi)biotroph resistance but enhances plant defenses towards necrotrophic pathogens (Fu and Wang 2011). In compliance with this concept, auxin and more specifically indole acetic acid (IAA), also act as virulence factors of the (hemi)biotrophic rice pathogens *M. oryzae*, *Xoo* and *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*), causal agent of bacterial leaf streak disease (Ding et al. 2008; Domingo et al. 2009; Fu et al. 2011). Like many other microbes, these pathogens produce and secrete IAA themselves and also increase IAA biosynthesis and signaling upon infection (Fu et al. 2011). In plants, auxin levels are regulated in part through negative feedback by a group of auxin-inducible GH3 (Gretchen Hagen 3) family genes that catalyze the conjugation of IAA to various amino acids. Unlike in Arabidopsis where three distinct groups of GH3 enzymes have been identified, only group I and II are present in rice (Westfall et al. 2010). Up to now, three group II GH3 enzymes have been functionally characterized in rice, namely OsGH3.1, OsGH3.2 and OsGH3.8. Consistent with IAA promoting hemibiotroph susceptibility, rice transformants overexpressing these enzymes displayed reduced levels of IAA and enhanced resistance to *M. oryzae*, *Xoo* and *Xoc* (Ding et al. 2008; Fu et al. 2011; Domingo et al. 2009).

In Arabidopsis, auxin is widely believed to antagonize SA-mediated defenses against biotrophic pathogens (Zhang et al. 2007; Truman et al. 2010). Two recent papers however suggest that auxin can also promote *Pst* growth and disease development in Arabidopsis via a mechanism independent of suppression of SA action (Mutka et al. 2013; González-Lamothe et al. 2012). These findings echo previous studies in rice where IAA-induced (hemi)biotroph susceptibility was found to be independent of SA and JA (Ding et al. 2008; Fu et al. 2011). Instead, it has been proposed that pathogen-triggered IAA promotes susceptibily by inducing the expression of cell wall-loosening expansins, thereby facilitating pathogen entry and allowing increased nutrient leakage. Notably, *OsGH3.2*–overexpressing plants not only exhibit decreased IAA levels, but also produce less ABA, which may contribute to the resistance to *M. oryzae* and *Xoo* (Du et al. 2012). In addition, these plants are also more tolerant to cold and oxidative stresses, providing a genetic strategy for breeding rice with broad-spectrum stress tolerance using *GH3* family genes. In this context, it will be particularly interesting to assess whether auxin promotes resistance to necrotrophic rice pathogens, as was previously shown in Arabidopsis (Llorente et al. 2008).

Cytokinins

Cytokinins (CK) are a group of N^6-substituted adenine derivatives that orchestrate myriad growth and developmental processes in plants. As one of the latest hormones to be linked with immunity, the precise role of CKs in plant immunity remains to be fully elucidated. Historically, CKs are associated with disease symptoms and morphological anomalies, such as fasciation, senescence, and the formation of galls, tumors and so-called 'green islands'. Many fungal and bacterial pathogens can produce CK themselves and/or increase CK synthesis in plants (Walters et al., 2008; Siemens et al., 2006). Although these observations indicate a role of CK in promoting pathogen virulence, recent work has revealed that CKs can also augment plant immunity. For example, CK has been demonstrated to render Arabidopsis more resistant against a fairly broad range of pathogens exhibiting different lifestyles (Argueso et al. 2012; Naseem et al. 2012; Swartzberg et al. 2008; Groaykinsky et al. 2011) suggested that the levels of CK are important in shaping plant immunity. In this study, low concentrations of the cytokinin benzyl adenine (BA) promoted susceptibility of Arabidopsis to the biotroph *Hyaloperonospora arabidopsidis*, whereas high BA concentrations enhanced disease resistance by priming the SA defense pathway (Argueso et al. 2012). Biochemical analyses revealed that this positive CK-SA crosstalk is mediated through a direct interaction between the CK-activated transcription factor ARR2 and the SA response factor TGA3, resulting in potentiated promoter binding of TGA3 and increased expression of SA-dependent defense genes (Choi et al. 2011, 2010). Meanwhile, SA feedback-inhibits CK signaling, which may serve to fin-tune the effect of CK in plant immunity (Argueso et al. 2012). In tobacco, however, a different mechanism appears to be operative. In this plant species, CK enhances resistance to *P. syringae* pv. *tobacco* independently of SA, indicating that nuanced, species-specific mechanisms underlie CK's immune-regulatory function (Groaykinsky et al. 2011).

Although exogenous application of CKs at low and high concentrations did not alter *M. oryzae* progression in rice, CK was found to synergistically interact with SA to activate *PR* genes in detached leaf assays (Jiang et al. 2013). Paradoxically, *M. oryzae* secretes CK itself and activates CK signaling in infected leaves, which might facilitate *M. oryzae* infection by increasing the sink strength of infected tissues (Jiang et al. 2013). Similarly, we found CK to negatively regulate rice resistance to *Xoo* (Chapter 6). Intriguingly, this CK-induced susceptibility is independent of SA signaling, but seems to be linked with activation of the plant growth-promoting protein Target of Rapamycin (TOR) (Chapter 6). It is hypothesized that CK-induced TOR activity tilts the plant's growth-versus-defense balance towards growth,

thereby reducing the amount of resources available for the plant to mount an effective immune response.

Brassinosteroids

Brassinosteroids are a unique group of plant steroidal hormones that play pivotal roles in cell expansion and division, differentiation and reproductive development. Although BRs have long been seen as mainly positive players in plant immunity, recent findings in both dicots and rice suggest a more complex situation, with positive, negative as well as neutral BR effects being reported that are seemingly independent of either the plant species or type of pathogen involved (De Bruyne et al. 2014). In rice, for instance, BR promotes resistance to the hemibiotrophic leaf pathogens *Xoo* and *M. oryzae*, while inducing susceptibility to the biotrophic root-knot nematode *Meloidogyne graminicola* and the necrotrophic oomycete *Pythium graminicola* (Nahar et al. 2013; De Vleesschauwer et al. 2012; Nakashita et al. 2003).

Accordingly, recent studies have revealed a wide variety of underpinning mechanisms, ranging from orchestration of oxidative metabolism and secondary metabolite production to modulation of PAMP perception and ensuing PTI signaling (De Bruyne et al. 2014). Depending on the relative hormone concentration among others and their effect on the LRR-receptor like kinase BAK1, BRs can act both antagonistically and synergistically with PTI responses (Belkhadir et al. 2012; Albrecht et al. 2012; Lozano-Durán et al. 2013; Shi et al. 2013). In addition, BRs have been found to cross-communicate with a range of other hormones, including SA, JA, ABA, auxins and GA (De Bruyne et al. 2014). Consistent with its apparent pluriform role in directing plant immune responses, the nature and direction of this BR hormone crosstalk can vary widely. For instance, whereas there is evidence for synergistic BR-SA crosstalk in Arabidopsis, previous studies with rice revealed that BR enhances resistance to *Xoo* and *Mo* in an SA-independent manner while disabling SA-mediated defenses against root pathogens (Divi et al. 2010; De Vleesschauwer et al. 2012; Nakashita et al. 2003). Much like GAs and CKs, BRs thus seem to play ambiguous roles in the plant defense network, the effect of which may depend not only on the pathogen's lifestyle and infection strategy, but also on speciliazed features of each interaction.

Gibberellins and DELLA proteins

GAs are a class of tetracyclic diterpenoid hormones that affect nearly every aspect of plant growth and development. According to current concepts, GAs promote plant growth by inducing the degradation of a class of nuclear proteins, called DELLAs. Arabidopsis mutants lacking four of the five DELLA proteins showed attenuated induction of the JA marker gene *PDF1.2*, resulting in enhanced susceptibility to the necrotrophic fungus *Alternaria brassicicola* (Navarro et al. 2008). In contrast, the same mutants exhibited increased levels of resistance to biotrophic *Pst* accompanied with elevated levels of SA (Navarro et al. 2008). On the basis of these and other findings, it was proposed that DELLAs modulate the strength of SA/JA signaling during plant immunity, promoting JA perception and/or signaling, and repressing SA biosynthesis and signaling. Accordingly, pretreatment with GA restricts JA signaling, resulting in enhanced SA signaling and increased biotroph resistance (Navarro et al. 2008).

In rice, however, strikingly different results have been obtained in that exogenous GA was found to enhance susceptibility against the hemibiotrophic pathogens *Xoo* and *M. oryzae*. Moreover, ectopic expression of a GA-deactivating enzyme designated Elongated Uppermost Internode (EUI) significantly reduced rice SA and JA levels and enhanced resistance to the latter pathogens, whereas EUI loss-of-function mutations led to increased susceptibility (Yang et al. 2008). Other mutants deficient in biosynthesis or perception of GA showed similar gain-of-resistance phenotypes when challenged with either *Xoo* or *M. oryzae* (Tanaka et al. 2006; De Vleesschauwer et al. 2013). On the other hand, GA was shown to be a positive player in resistance against the necrotrophic root pathogen *P. graminicola* (De Vleesschauwer et al. 2012). Therefore, opposite to the situation in Arabidopsis, rice GA signaling appears to induce susceptibility to (hemi)biotrophic pathogens and resistance to necrotrophs.

Although much remains to be discovered about the precise mechanisms via which GA and DELLAs modulate plant immunity, recent studies have implicated DELLAs in a variety of processes, including the regulation of oxidative and energy metabolism, cell wall development, and cytoskeleton architecture (De Bruyne et al. 2014). Moreover, evidence is accumulating that DELLA orchestrates plant immunity via competitive binding to JA ZIM-domain (JAZ) proteins, a family of JA signaling repressors. JAZ proteins bind and inhibit the activity of numerous TFs, including the key JA transcriptional activator MYC2 (Kazan and Manners 2013, 2012). Recently, three groups have shown that DELLAs compete with MYC2 for binding to JAZs, thereby releasing free MYC2 to activate JA-responsive gene expression and, hence, increase resistance to necrotrophic pathogens (Hou et al. 2010; Wild et al. 2012;

Yang et al. 2012). In the presence of GA, however, DELLAs are rapidly degraded, leading to inhibitory JAZ-MYC2 interactions and disruption of JA signaling. This so-called 'relief of repression' model not only elegantly explains how plants balance growth and defense responses, but also offers novel insights into how GA disables JA-mediated necrotroph resistance by degrading DELLAs and releasing JAZs to bind and inhibit MYC2.

Consistent with these findings in Arabidopsis, Yang et al. (2012a) demonstrated that SLR1, the only DELLA in rice, serves as a main target of JA-mediated growth inhibition and is required for full-scale activation of JA-responsive gene expression in rice. Moreover, preliminary findings by our lab revealed that SLR1 physically interacts with several rice JAZ proteins, suggesting that the core mechanism(s) underpinning DELLA-JA interactions are conserved in rice. More surprisingly, however, we also found SLR1 to amplify BTH- and pathogen-induced expression of SA-responsive marker genes (De Vleesschauwer et al., unpublished). Together, these data favor a model wherein SLR1 steers (hemi)biotroph resistance in rice by integrating and amplifying SA and JA signaling pathways. Considering that many other hormones affect SLR1 protein stability, either directly or indirectly, it thus seems that SLR1 is positioned at the intersection of various hormone pathways, acting as a main hub for signal crosstalk and pathway integration (De Bruyne et al. 2014).

Concluding remarks

The past decade has seen tremendous progress in our understanding of hormone defense signaling in Arabidopsis and other dicot plants. However, as illustrated throughout this review, the conceptual framework emerging from these studies does not always translate to monocot systems. While underscoring the importance of using alternative models systems, the unique complexities associated with defense networking in rice call for a re-evaluation of overly generalized defense models.

Contrary to the classic binary defense model with SA and JA playing opposite roles in biotroph and necrotroph resistance, respectively, innate immunity in rice appears to be controlled by a much more complicated signaling network that supports no clear dichotomy between the effectiveness of most hormone pathways and the overall infection biology of the invading pathogen. Most conspicuously, although hyperactivation of one can attenuate the other, synergistic SA-JA interactions seem to prevail in rice and the two hormones are effective against both hemibiotrophic and necrotrophic rice pathogens. Moreover, unlike in dicots, we are unaware of any reports showing negative effects of SA or JA on rice immunity.

Therefore, it is not unlikely that both hormones function as endogenous priming agents that amplify infection-induced defense reactions regardless of the lifestyle of the invading pathogen. In contrast, ET can have both positive and negative effects on rice disease resistance that are seemingly independent of the pathogen's parasitic habits. The impact of developmental hormones is equally complex. As was reported in Arabidopsis, auxin promotes susceptibility to hemibiotrophic rice pathogens, while CK signaling can cascade either to the detriment or the benefit of plant. GAs, on the other hand, appear to play opposite roles in rice and Arabidopsis, which may be explained by the ability of the rice DELLA protein SLR1 to integrate and amplify SA and JA signaling. Finally and consistent with their ambivalent role in dicot immunity, BRs and ABA can both promote and suppress rice immunity depending not only on the type of pathogen, but also on the type of tissue, and even spatial and temporal conditions.

Despite the recent progress, much remains to be learned about the role of hormones in the regulation of the rice defense signaling network. For instance, it is still unclear how SA and JA are perceived in rice and how their signaling pathways interact at the molecular level, there is little information available about the impact of viruses, insects and nematodes on the rice hormone infrastructure, and there is still much to be learned about the hormone intervention strategies used by rice pathogens to inflict disease. Moreover, few studies have investigated the spatiotemporal dynamics of a given hormone during rice-pathogen interactions and none addresses the kinetics and signature of the blend of hormones released upon pathogen attack. Finally, there is a paucity of knowledge on the molecular players orchestrating pathway crosstalk and signal integration in the rice signaling circuitry. Deepening our knowledge in this area is especially important since defining synergies and trade-offs may help identify appropriate contexts for the optimal deployment and commercial acceptance of hormone-based rice disease resistance.

Chapter 4

Abscisic acid (ABA) promotes susceptibility of rice towards bacterial leaf blight pathogen *Xanthomonas oryzae* pv. *oryzae* by suppressing salicylic acid-mediated defenses

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Abstract

The plant hormone abscisic acid (ABA) is involved in a wide variety of plant processes, including the initiation of stress-adaptive responses to various environmental cues. Recently, ABA also emerged as a central factor in the regulation and integration of plant immune responses, although little is known about the underlying mechanisms. Aiming to advance our understanding of ABA-modulated disease resistance, we have analyzed the impact, dynamics and interrelationship of ABA and the classic defense hormone salicylic acid (SA) during progression of rice infection by the leaf blight pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Consistent with ABA negatively regulating resistance to *Xoo*, we found that exogenously administered ABA renders rice hypersusceptible to infection, whereas chemical and genetic disruption of ABA biosynthesis and signaling, respectively, led to enhanced *Xoo* resistance. In addition, we found successful *Xoo* infection to be associated with extensive reprogramming of ABA biosynthesis and response genes, as well as increased endogenous ABA level, suggesting that ABA functions as a virulence factor for *Xoo*. Interestingly, several lines of evidence indicate that this immune-suppressive effect of ABA is due at least in part to suppression of SA-mediated defenses that normally serve to limit pathogen growth. Resistance induced by the ABA biosynthesis inhibitor fluridone, however, appears to operate in a SA-independent manner and is likely due to induction of non-specific physiological stress. Collectively, our findings favor a scenario whereby virulent *Xoo* hijacks the rice ABA machinery to cause disease and highlight the importance of ABA and its crosstalk with SA in shaping the outcome of rice-*Xoo* interactions.

Introduction

As sessile organisms, plants are continuously threatened by a suite of biotic and abiotic stress factors. Many of the defense mechanisms employed to counteract these stresses are controlled by an array of signal transduction pathways within which plant hormones function as key signaling molecules. Salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are the classic immunity hormones, while the importance of other small-molecule hormones including auxin, brassinosteroids (BR), gibberellic acid (GA), cytokinins (CK) and abscisic acid (ABA) is now gaining momentum (Asselbergh et al. 2008b; Bari and Jones 2009; Pieterse et al. 2012; Robert-Seilaniantz et al. 2011; De Vleesschauwer et al. 2012). Upon infection, plants produce a highly specific blend of hormonal alarm signals, resulting in the activation of disparate sets of attacker-specific immune responses (De Vos et al. 2005). SA, for instance, is commonly associated with defense against biotrophic pathogens, whereas

necrotrophic pathogens are generally believed to be deterred by JA/ET-driven defenses (Pieterse et al. 2012).

Yet, rather than driving independent, linear routes of signal processing, hormones function within complex regulatory networks that connect the different pathways, enabling each to assist or antagonize the others. This interplay or so-called 'crosstalk' between individual hormones is thought to confer flexibility to the immune response, allowing the plant to adjust its inducible defense arsenal to the type of attacker encountered (Spoel and Dong 2008). Exciting new developments, however, indicate that crosstalk may also allow successful pathogens to manipulate the plant's defense signaling network for their own benefit by shutting down effective defenses (Pieterse et al. 2009). A classic example reflecting this situation is the production by some *Pseudomonas syringae* strains of a phytotoxin called coronatine that structurally resembles JA derivatives, including JA-isoleucine (Staswick 2008). Coronatine is actively secreted in the host and hyperactivates JA signaling, resulting in suppression of effectual SA-mediated defenses and increased disease susceptibility (Brooks et al. 2005; Cui et al. 2005).

Contrary to the relative wealth of information with respect to SA, JA and ET serving as defense regulators, the role of abscisic acid (ABA) in plant innate immunity is still poorly understood. Most comprehensively studied for its role in plant responses to environmental stresses, ABA has only recently emerged as a pivotal determinant in the outcome of plant-pathogen interactions (Asselbergh et al. 2008b; Cao et al. 2011; Ton et al. 2009). In some interactions, ABA positively influences disease outcomes. For instance, ABA primes for callose deposition and thereby enhances basal defense against the powdery mildew fungus *Blumeria graminis* and the necrotrophic fungus *Alternaria brassicicola*, and also activates JA-mediated resistance against the oomycete *Pythium irregular* (Ton and Mauch-Mani 2004; Flors et al. 2008). In addition, ABA is required for stomatal closure, which as part of the SA-mediated pre-invasion immune response, is a major barrier against bacterial invasion (Melotto et al. 2006). In most cases, however, ABA acts as a negative regulator of disease resistance with inhibition of ABA biosynthesis and/or signal transduction commonly resulting in enhanced disease resistance to a wide variety of bacterial, fungal and oomycete pathogens exhibiting distinct parasitic habits (Grant et al. 2007; Adie et al. 2007; Audenaert et al. 2002; Achuo et al. 2006; Cahill and Mohr 2007; Asselbergh et al. 2008a; Yasuda et al. 2008; Ho et al. 2013). The importance of ABA in plant immunity is underscored by the ability of pathogens to either produce ABA themselves and/or to modify ABA biosynthesis and signaling *in planta*. In Arabidopsis, for instance, it was shown that *P. syringae* hijacks the ABA biosynthetic and response machinery to cause disease, indicating that ABA is a

susceptibility factor for this bacterium (Grant et al. 2007). Similarly, Jiang et al. (2010) reported transiently elevated ABA titers in rice plants attacked by the blast fungus *Magnaporthe oryzae* (Jiang et al. 2010). Current concepts suggest that this infection-induced ABA enables pathogens to tap into the plant's defense signaling circuitry and interfere with host immunity. In support of this notion, there is ample evidence demonstrating the ability of ABA to interfere either directly or indirectly with the SA-JA-ET backbone of the plant defense circuitry (Asselbergh et al. 2008b; Pieterse et al. 2012; Anderson et al. 2004; Fan et al. 2009). Additionally, ABA has been proposed to counteract GA-controlled defenses by promoting the stability of DELLA proteins that inhibit GA signaling (Grant and Jones 2009), while exciting new molecular insights connect ABA also to CK-mediated stress responses (Peleg et al. 2011; Tran et al. 2007; Wang et al. 2011; Nishiyama et al. 2011).

Rice is one of the most important staple food crops worldwide, providing the bulk of the daily caloric intake for no less than 3 billion people living in tropical and subtropical Asia. However, despite its emergence as a pivotal model for studying innate immunity in monocotyledonous plants (Seo et al. 2011), studies addressing the role of plant hormones, and especially ABA, in the rice defensive machinery are scarce. In previous work, we have shown that ABA enhances basal resistance against the rice brown spot pathogen *Cochliobolus miyabeanus* by preventing the fungus from hijacking the ET pathway (De Vleesschauwer et al. 2010). Interestingly, these ABA and ET-provoked effects are reverse of those against the blast fungus *M. oryzae*. In this pathosystem, ABA is thought to condition susceptibility via suppression of effectual ET- and SA-mediated defenses (Jiang et al. 2010; Bailey et al. 2009). In contrast, molecular information regarding the role of ABA in bacterial leaf blight (BLB) disease is still elusive. BLB, caused by the gram-negative bacterium *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is one of the most widespread and destructive rice diseases, causing annual yield losses up to 60% (Niño-Liu et al. 2006). Aiming to further decipher the molecular underpinnings of ABA-modulated rice immunity, we sought to determine the impact, dynamics and interrelationship of ABA with other hormones during progression of *Xoo* infection. Through genetic, physiological and pathological analyses, we show that ABA suppresses basal immunity of rice against virulent *Xoo* and likely functions as a virulence factor for the bacterium. Moreover, we demonstrate that ABA induces susceptibility of rice to *Xoo* by attenuating effectual SA defenses and provide evidence that this ABA-SA antagonism occurs downstream of SA biosynthesis, but upstream or at the level of the master defense regulators *OsNPR1* and *OsWRKY45*.

Results

ABA negatively regulates resistance to *Xoo*

In a first attempt to unravel the role of ABA in the rice-*Xoo* pathosystem, we examined the effect of exogenous hormone application on subsequent pathogen inoculation. To this end, leaves of 6-week-old *indica* cultivars IRBB3 and IRBB13 were sprayed until runoff with a 100 µM ABA solution and, three days later, inoculated with *Xoo* strain PXO99 using the leaf-clipping method (Kauffman et al. 1973). PXO99 is virulent to IRBB3, but avirulent to IRBB13 which harbors the recessive *R* gene *xa13* (Chu et al. 2006). In all bioassays, disease development was routinely monitored at 14 dpi by recording the length of the water-soaked lesions characteristic of leaf blight disease. As shown in Figures 4.1A and 4.1C, exogenous ABA application significantly lowered basal disease resistance in the susceptible IRBB3 background, with average lesions of 18 cm on ABA-treated plants compared to control, non-treated plants, which displayed average lesion lengths of 12 cm. In contrast, resistant IRBB13 seedlings inoculated with PXO99 displayed only marginal symptom development (lesions shorter than 1 cm) and ABA pretreatment appeared to have little or no effect in this background.

To further characterize the effect of ABA on *Xoo* immunity, we next assessed the impact of *in planta* ABA levels. Due to the lack of well-characterized ABA-deficient mutants in rice, a pharmacological approach was followed whereby hydroponically grown IRBB3 and IRBB13 plants were supplied for 6 days with the ABA biosynthesis inhibitor fluridone (Watanabe et al. 2001), and subsequently inoculated with PXO99. Corroborating our results with exogenous ABA, fluridone application substantially reduced disease severity in susceptible IRBB3, but failed to exert an additive effect on the already high levels of *Xoo* resistance in IRBB13 (Figure 4.1A). Importantly, fluridone had no significant effect on *in vitro* growth of PXO99 (data not shown), demonstrating the involvement of plant-mediated responses.

Bacterial growth correlated well with lesion length development (Figure 4.1B). At 16 dpi, PXO99 titers reached approximately 2×10^{10} CFU/leaf in ABA-pretreated IRBB3, a greater than 100-fold increase compared to non-treated control IRBB3. In fluridone-treated IRBB3, however, PXO99 grew 10-fold less than in the controls with populations leveling off to fewer than 2×10^{7} cfu/leaf. In contrast, no significant differences between treatments could be observed in resistant IRBB13 where PXO99 populations reached approximately 8×10^{6} cfu/leaf within 16 dpi. Together with the results from the lesion length measurements, these data strongly suggest that ABA suppresses basal immunity to *Xoo* and, hence, acts as a negative regulator of BLB resistance.

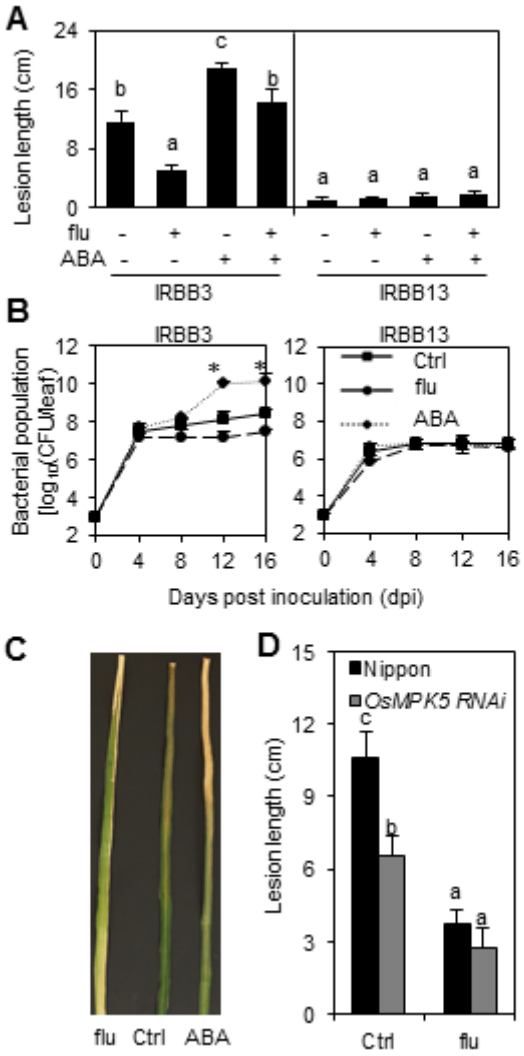

Figure 4.1. Effect of exogenous and endogenous abscisic acid (ABA) on bacterial leaf blight (BLB) development in rice. (A). Susceptible IRBB3 and resistant IRBB13 plants were pretreated with ABA (100 µM) and/or the ABA inhibitor fluridone (flu; 0.4 µM) for 3 and 6 days, respectively. Fifth and sixth stage leaves were inoculated with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) strain PXO99 using the standard leaf-clipping method. Fourteen days post inoculation (dpi), disease was evaluated by measuring the length of the water-soaked BLB lesions. Data are means ± SE of at least 10 plants. Different letters indicate statistically significant differences (Mann-Whitney: n ≥20; a=0.05). (B). Effect of ABA (100 µM) and fluridone (0.4 µM) on PXO99 titers in susceptible IRBB3 and resistant IRBB13. Data are means ± SE of three biological replicates. Asterisks indicate statistically significant differences compared to control treatments (LSD; n=3; a=0.05). (C). Symptom development on Ctrl, ABA or fluridone-pretreated IRBB3 leaves at 14 dpi. (D). Effect of fluridone (0.4 µM) on BLB development in *OsMPK5* RNAi and WT Nipponbare plants. Data are means ± SE of at least 10 plants. Different letters indicate statistically significant differences (Mann-Whitney: n ≥ 20; a=0.05). All experiments were repeated at least twice with similar results.

To substantiate this hypothesis, we quantified the level of basal and fluridone-inducible *Xoo* resistance in plants silenced for the MAP kinase gene *OsMPK5*. One of the better studied MAP kinases in rice, *OsMPK5* has been shown to function as a positive regulator of ABA

signaling in rice (Xiong and Yang 2003). Accordingly, *OsMPK5* RNAi plants are partially ABA-insensitive and display reduced expression of ABA-responsive genes (Bailey et al. 2009). As shown in Figure 4.1D and consistent with previous results (Seo et al. 2011), non-treated *OsMPK5* RNAi plants were significantly less susceptible to PXO99 than similarly treated wild-type plants, while fluridone application was equally effective in both genotypes, further confirming the negative impact of ABA on basal *Xoo* resistance.

Temporal dynamics of ABA biosynthesis and signaling in response to *Xoo* inoculation

To gain more insight into the mechanism(s) of ABA-induced *Xoo* susceptibility, we monitored the steady-state mRNA levels of several ABA biosynthetic and ABA responsive genes in control and ABA-pretreated IRBB3 leaves at various times after inoculation with PXO99. As shown in Figures 4.2A-B, expression of the ABA biosynthetic genes *OsNCED3* and *OsNCED4* remained static at early time points but increased steadily from 4 dpi and peaked at 8 dpi at approximately 10 and 150 times the levels found in non-inoculated controls, respectively. Interestingly, transcription of the ABA-responsive genes *OsLip9* and *OsRab16* mirrored the profiles observed for *OsNCED3* and *OsNCED4*, these genes being strongly upregulated at 4 and 8 dpi. Comparing control and ABA-treated samples at 0 dpi, no major differences could be observed for both *OsNCED4* and *OsLip9*. Expression of *OsNCED3* and *OsRab16*, on the other hand, was significantly higher in ABA-treated samples compared to control plants. In a similar vein, ABA application strongly boosted the expression of *OsNCED4* and both ABA-responsive genes following *Xoo* attack, especially at 8 dpi (Figures 4.2B-D).

In a set of parallel experiments, we also studied the expression profiles of *OsNCED3*, *OsLip9* and *OsRab16* in response to fluridone application. In line with abovementioned results, expression of these genes responded strongly to *Xoo* infection from 4 dpi onward (Figures 4.3A-C), whereas fluridone application strongly alleviated this pathogen-induced activation. Thus, ABA pretreatment boosts basal and/or pathogen-induced expression of ABA-responsive genes and enhances susceptibility to *Xoo*, whereas fluridone inhibits ABA-responsive gene expression and increases resistance to *Xoo*. When considered together, these data indicate that successful *Xoo* infection is associated with extensive reprogramming of ABA biosynthesis and ABA responsive genes. Hence, we measured ABA contents in rice leaves after virulent *Xoo* infection using UPLC as described by Kojima et al (2009) (Kojima et al. 2009). In accordance with the gene expression, the ABA concentration in PXO99-infected T65 leaves was almost doubled at 4 dpi, and increased about 20 folds at 8 dpi compared with that in non-infected leaves (Table 4.1). Moreover, in conjunction with earlier findings

that ABA titers rise to a higher extent in compatible versus incompatible rice-*Xoo* interactions (Liu et al. 2012), these observations raise the possibility that virulent *Xoo* may hijack the rice ABA pathway to induce a state of susceptibility.

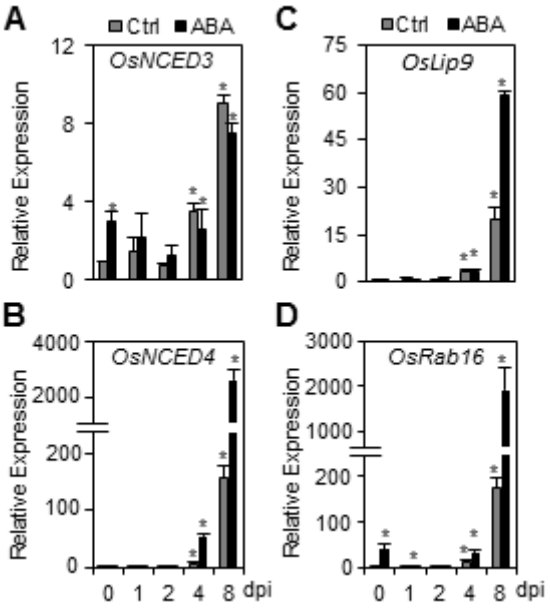

Figure 4.2. Dynamics of ABA pathway in response to virulent *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) infection. (A) through (D). Effect of ABA pretreatment on ABA-biosynthesis (*OsNCED3, OsNCED4*) and ABA-responsive genes (*OsLip9* and *OsRab16*) in IRBB3 leaves inoculated with *Xoo* strain PXO99. For details on ABA pretreatment and *Xoo* inoculation, see legend to Figure 4.1. Transcript levels were normalized using eukaryotic elongation factor *eEF1a* as an internal reference and, for each treatment, expressed relative to the normalized expression levels in mock-inoculated control plants at the appropriate time point. Data are means ± SD of two technical and two biological replicates from a representative experiment, each biological replicate representing a pooled sample from 3 individual plants. Two sets of independent experiments were carried out with similar results. Asterisks indicate statistically significant differences per treatment compared to either control (0 dpi) or mock-treated samples (1, 2, 4 and 8 dpi).

Table 4.1 Quantification of ABA in T65 leaves infected with PXO99. Data are means (pmol/g fresh weight) ± SD of three biological replicates, each biological replicate representing a pooled sample from 3 individual plants. dpi: day post inoculation. Asterisks indicate statistically significant differences compared to the Mock at the same timepoint (T-test, n=3, α =0.05). dpi: day post inoculation.

dpi	Mock	Infected
0	187.99±15.98	
1	334.38±68.08	355.4±7.99
2	215.88±23.32	260.25±22.52
4	197.11±31.8	398.08±90.25
8	405.67±51.78	8074.42±821.9*

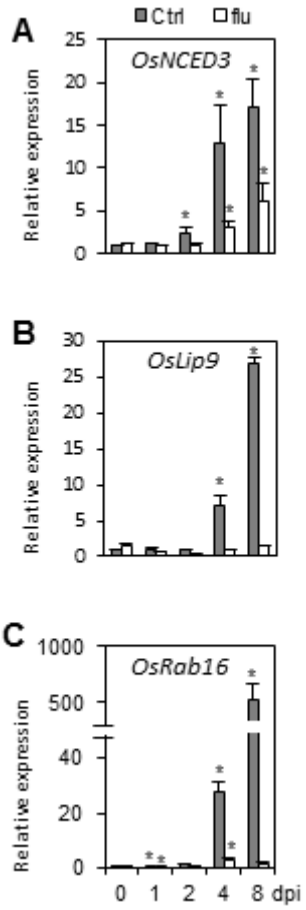

Figure 4.3. Fluridone suppresses pathogen-induced transcription of ABA biosynthesis and response genes. (A) through (C). Relative expression of ABA biosynthesis and responsive genes, *OsNCED3*, *OsLip9* and *OsRab16*, in control (Ctrl) and fluridone-pretreated (0.4 µM) IRBB3 leaves inoculated with PXO99. Transcript levels were normalized using eukaryotic elongation factor *eEF1a* as an internal reference and expressed relative to the normalized expression levels in mock-inoculated control plants at the appropriate time point. Data are means ± SD of two technical and two biological replicates from a representative experiment, each biological replicate representing a pooled sample from 3 individual plants. Two sets of independent experiments were carried out with similar results. Asterisks indicate statistically significant differences per treatment compared to either control (0 dpi) or mock-treated samples (1, 2, 4 and 8 dpi).

ABA negatively regulates *Xoo* resistance by attenuating SA-mediated defenses

In Arabidopsis, ABA has been repeatedly shown to negatively regulate plant disease resistance by antagonizing the SA signaling pathway (Cao et al. 2011; Yasuda et al. 2008; Fan et al. 2009). Similarly, Jiang et al. (2010) reported that ABA compromises resistance of rice to fungal blast disease by suppressing effective SA-mediated defense responses. To further confirm antagonistic crosstalk between ABA and SA in rice and expand the scope of the investigation, we assessed the effect of single and combined hormone treatments on the expression of ABA and SA marker genes. For this purpose, leaf blade segments of 6-week–

old IRBB3 seedlings were incubated for 8 h in aqueous solutions of the respective hormones, and subsequently analyzed by quantitative RT-PCR. As shown in Figure 4.4A, single ABA treatment resulted in strong activation of the ABA marker gene *OsLip9*, while co-application of ABA with SA alleviated this ABA-induced *OsLip9* expression, indicating negative crosstalk in the direction of SA damping ABA action. However, consistent with bidirectional SA-ABA crosstalk, we also found ABA to impact the expression of both *OsNPR1* and *OsWRKY45*, two master regulatory proteins that control distinct branches of the SA signaling cascade in rice (Yuan et al. 2007; Shimono et al. 2007). Expression of *OsWRKY45* was activated in response to SA, whereas ABA suppressed both basal and SA-inducible *OsWRKY45* expression (Figure 4.4B). In contrast but consistent with previous reports on detached leaf segments (Jiang et al. 2010), expression of *OsNPR1* was barely responsive to exogenous SA, though it was still markedly inhibited by ABA (Figure 4.4C).

Having confirmed negative SA-ABA signal interactions in rice, we next sought to assess the significance of this antagonism in shaping the outcome of rice-*Xoo* interactions. To this end, leaves of 6-week-old IRBB3 were sprayed with 100 µM ABA and/or 500 µM SA and three days later, inoculated with virulent PXO99. As shown in Figure 4.5D, exogenous ABA treatment significantly enhanced disease susceptibility, whereas SA application rendered plants more resistant to subsequent PXO99 inoculation. Moreover, co-application with SA discounted the disease-promoting effect of single ABA treatments, suggesting that ABA may govern susceptibility to *Xoo* at least in part by suppressing effectual SA-mediated defenses.

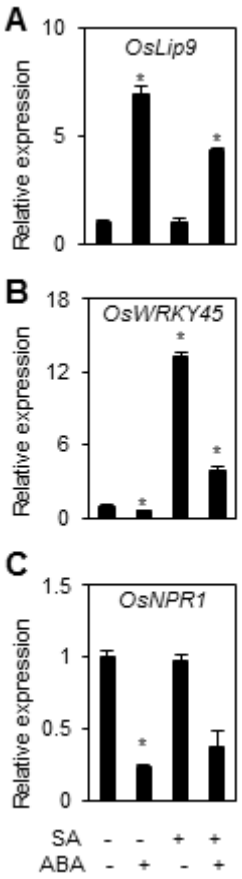

Figure 4.4. Cross-talk experiments demonstrating mutual antagonism between ABA and SA. IRBB3 leaf segments were incubated for 8 h in aqueous solutions containing 50 µM ABA and/or 500 µM SA and subsequently tested for expression of the ABA responsive gene *OsLip9* and SA marker genes *OsNPR1* and *OsWRKY45*. Transcript levels were normalized using eukaryotic elongation factor *eEF1a* as an internal reference and for each treatment expressed relative to the normalized expression levels in non-treated control plants. Data are means ± SD of two technical and two biological replicates from a representative experiment, each biological replicate representing a pooled sample from 13 individual plants. The experiment was repeated once with similar results. Asterisks indicate statistically significant differences compared control, non-treated samples.

To test this hypothesis, we monitored the temporal expression patterns of three SA regulatory genes in control and ABA-treated IRBB3 leaves following PXO99 infection. Besides *OsWRKY45* and *OsNPR1*, these genes included *OsWRKY13*, a well-characterized transcription factor gene functioning upstream of *OsWRKY45* and *OsNPR1* (Qiu et al. 2008, 2007). Consistent with the expression profiles reported in other studies (Yuan et al. 2007; Qiu and Yu 2009; Ryu et al. 2006; Tao et al. 2009), expression of *OsWRKY45* and *OsNPR1* responded only weakly to *Xoo* inoculation (Figure 4.5A-B). However, both genes were several-fold down-regulated in pathogen-inoculated leaves pretreated with ABA. Interestingly, ABA-mediated suppression of *OsWRKY45* was evident at 4 and 8 dpi only, which is in line with the upregulation of ABA biosynthesis and ABA signaling genes at these

time points. In contrast, expression of *OsWRKY13* was not responsive to ABA treatment at any time point (Figure 4.5C), suggesting that ABA antagonizes SA-mediated *Xoo* resistance downstream of *OsWRKY13*. This notion was further supported by the different effects of ABA pretreatment on BLB development in transgenic rice lines overexpressing *OsNPR1* and *OsWRKY13*. Consistent with previous studies (Qiu et al. 2008; Yuan et al. 2007), both *OsNPR1*-OX and *OsWRKY13*-OX lines exhibited increased resistance to *Xoo* compared to the respective wild-types (Figure 4.5E-F). However, while ABA application significantly promoted disease development in both WT and *OsWRKY13*-OX backgrounds, overexpressing *OsNPR1* fully blocked ABA-inducible *Xoo* susceptibility. Collectively, these data further confirm mutually antagonistic SA-ABA crosstalk during leaf blight infection and strengthen the hypothesis that ABA suppresses SA defenses downstream of *OsWRKY13* but upstream of *OsNPR1*.

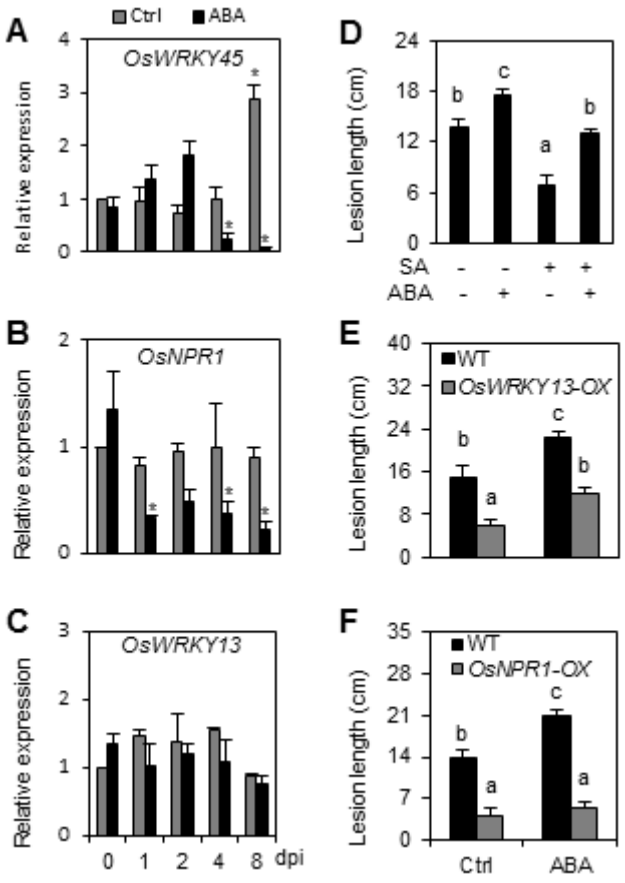

Figure 4.5. ABA counteracts SA-mediated defenses to *Xoo*. (A) through (C). Expression of SA marker genes *OsWRKY45*, *OsNPR1* and *OsWRKY13* in control (Ctrl) and ABA pretreated IRBB3 leaves inoculated with PXO99. Transcript levels were normalized using eukaryotic elongation factor *eEF1a* as an internal reference and for each treatment expressed relative to the normalized expression levels in mock- inoculated control plants at the appropriate time point. Data are means ± SD of two technical and two biological replicates from a representative experiment, each biological replicate representing a pooled sample from 3 individual plants. Asterisks indicate statistically significant differences per treatment compared to either control (0 dpi) or mock-treated samples (1, 2,

4 and 8 dpi). (D). Effect of single and combined pretreatment with ABA (100 µM) and/or SA (500 µM) on BLB development in susceptible IRBB3 plants. Lesions were measured 14 days after inoculation with PXO99. Data are means ± SE of at least 10 plants. Different letters indicate statistically significant differences (Mann-Whitney; n ≥20; a=0.05) (E) and (F). Effect of exogenous ABA treatment (100 µM) on BLB development in *OsNPR1*-OX and *OsWRKY13*- OX lines and their respective WT Taipei and Mudanjiang. Data are means ± SE of at least 10 plants. Different letters indicate statistically significant differences (Mann-Whitney; n ≥20; a=0.05). Repetition of experiments led to results similar to those shown.

Fluridone-inducible *Xoo* resistance is independent of SA

The observation that ABA induces *Xoo* susceptibility, at least in part, by antagonizing the SA pathway prompted us to check whether ABA-lowering fluridone induces resistance by de-repressing SA-mediated immune responses. To address this hypothesis, we initially checked the impact of fluridone application on the expression of the SA marker genes *OsWRKY45*, *OsNPR1* and *OsWRKY13* in IRBB3 leaves infected with PXO99. Consistent with Figures 4.5A-C, expression of *OsWRKY45*, *OsNPR1* and *OsWRKY13* showed little changes in response to PXO99 inoculation (Figures 4.6A-C). However, unlike the situation in ABA-treated leaves, no major and/or consistent changes in gene expression could be noticed between control and fluridone-treated samples, suggesting that fluridone-mediated resistance is not reliant on the SA pathway.

To further probe whether fluridone operates in an SA-independent manner, we quantified the level of fluridone-inducible resistance in both SA-non accumulating *NahG* and *OsNPR1* RNAi lines. As shown in Figure 4.6D, *NahG* plants were significantly more sensitive to pathogen attack than corresponding wild-type seedlings, demonstrating the importance of SA biosynthesis in basal resistance to *Xoo*. SA accumulation, however, did not appear to be a prerequisite for fluridone-inducible resistance, as fluridone application was equally effective in WT Nipponbare and *NahG* plants, causing an approximate 50% reduction in basal disease susceptibility in both genotypes. Similarly, fluridone triggered high levels of resistance in both WT Taipei and *OsNPR1* RNAi plants, indicating that, unlike ABA, fluridone functions independently of *OsNPR1* (Figure 4.6E).

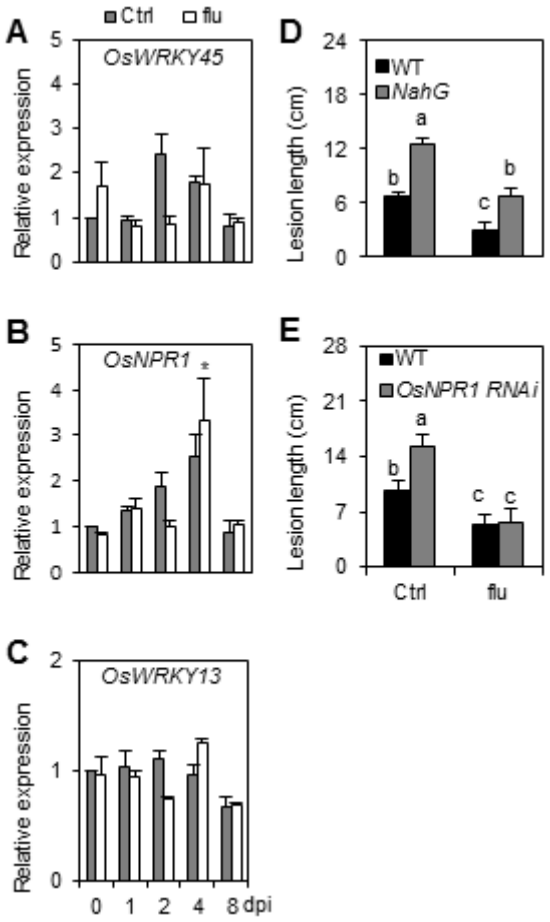

Figure 4.6. Fluridone induced *Xoo* resistance is independent of SA. (A) through (C). Transcript levels of the SA regulatory genes *OsWRKY45*, *OsNPR1* and *OsWRKY13* in control and fluridone-treated (0.4 mM) IRBB3 leaves inoculated with PXO99. Data are means ± SD of two technical and two biological replicates, each biological replicate representing a pooled sample from 3 individual plants. Asterisks indicate statistically significant differences per treatment compared to either control (0 dpi) or mock-treated samples (1, 2, 4 and 8 dpi). (D) and (E). Effect of fluridone (0.4 µM) on BLB development in *OsNPR1* RNAi and *NahG*-expressing lines and their respective WT Taipei and Nipponbare. Data are means ± SE of at least 10 plants. Different letters indicate statistically significant differences (Mann-Whitney; n ≥20; a=0.05). Repetition of experiments led to results similar to those shown.

Discussion

Bacterial leaf blight (BLB), caused by the gram-negative bacterium *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is one of the most devastating rice diseases owing to its widespread distribution and high pathogenic variability. However, despite the accumulated wealth of genetic and molecular resources in rice and the identification of over 30 major resistance genes for BLB (Niño-Liu et al. 2006), surprisingly little is known about the hormone signaling pathways underpinning disease and resistance in the rice-*Xoo* pathosystem. Previously, Ding et al. (2008) showed that auxin promotes susceptibility to *Xoo* through induced

expression of cell wall-loosening expansins. In contrast, SA and JA act as positive regulators of immunity against *Xoo* (Qiu et al. 2008; Yuan et al. 2007; Tao et al. 2009), while GA and ET are reported to suppress BLB resistance through yet to be defined mechanisms (Shen et al. 2011; Yang et al. 2008). In adding to this list, our results uncover ABA as an additional negative regulator of rice-*Xoo* interactions. Moreover, our findings highlight the importance of bidirectional ABA-SA signal interplay in determining the outcome of rice-*Xoo* interactions and suggest that virulent strains of *Xoo* exploit ABA to subdue the rice innate immune system and promote disease development.

ABA negatively regulates rice immunity to *Xoo*

Contrary to the well-characterized role of ABA in plant adaptive responses to abiotic stress (Fujita et al. 2006), its contribution to plant disease resistance is relatively poorly understood, and even contentious. Whereas the majority of reports indicate that ABA suppresses pathogen defense responses (Ton et al. 2009), others have pinpointed a positive role of ABA in plant immunity (Ton and Mauch-Mani 2004; De Vleesschauwer et al. 2010; Adie et al. 2007). Recent studies also found that the role of ABA in modulating disease resistance may depend not only on pathogen lifestyle but also on temporal and spatial conditions, indicating that complex nuanced mechanisms underlie ABA modulation of plant immunity (Asselbergh et al. 2008b; Cao et al. 2011). Under our experimental conditions, exogenous ABA application significantly increased rice susceptibility to virulent *Xoo* (Figure 4.1A), while lowering basal ABA levels by applying the ABA biosynthesis inhibitor fluridone or genetic disruption of ABA signaling in *OsMPK5* RNAi plants led to reduced disease development (Figures 4.1A-B). Similar to what was previously reported for the leaf blast fungus *M. oryzae* (Jiang et al. 2010; Koga et al. 2004; Yazawa et al. 2012), ABA thus seems to act as a negative regulator of rice immunity to *Xoo*.

Interestingly, both exogenous ABA treatment and fluridone application failed to alter lesion length development and bacterial growth in IRBB13 plants carrying the recessive *R* gene *xa13*, suggesting that ABA predominantly affects basal defense responses against *Xoo*. However, care should be taken when interpreting these data. Recently, Mang et al. (2012) mechanistically connected ABA to *R* protein-mediated immunity by demonstrating that ABA deficiency in Arabidopsis promotes defense responses at high temperatures through enhancing the nuclear accumulation and activity of the resistance proteins *SNC1* and *RPS4* (Mang et al. 2012). Consistent with this, exogenous ABA treatment was previously reported to compromise resistance to both virulent and avirulent blast fungus isolates, indicating that ABA negatively orchestrates both basal and *R* protein-mediated resistance against *M. oryzae*

(Jiang et al. 2010). Taking these facts into account, it is not inconceivable that ABA may play a role in *Xoo* resistance governed by *R* genes other than *xa13*. Additional bio-assays using *Xoo* strains with different genetic backgrounds and rice lines carrying distinct types of *R* genes will aid in deciphering the role, if any, of ABA in regulating *R*-gene mediated resistance to *Xoo*.

The importance of ABA in determining pathological outcomes is underscored by the efforts pathogens undertake to tap into the host ABA biosynthesis and signaling infrastructure. Recent studies have demonstrated the direct manipulation of ABA biosynthesis and signaling by bacterial type III effectors as a virulence strategy for *P. syringae* and *X. campestris* pv. *campestris* (Grant et al. 2007; Ho et al. 2013; De Torres Zabala et al. 2009; Goel et al. 2008). Moreover, in addition to modifying plant ABA biosynthesis, some phytopathogenic organisms, including the fungal pathogens *M. oryzae*, *Botrytis cinerea* and *Rhizoctonia solani*, are able to produce and secrete ABA themselves (Oritani and Kiyota 2003; Siewers et al. 2006). Since there is no compelling evidence supporting the role of ABA in the physiology of these pathogens, it is likely that pathogens have evolved ABA biosynthetic machinery to trigger ABA signaling at infection sites and dampen plant immunity (Ton et al. 2009). Previously, Liu et al (2012) demonstrated that rice plants responding to *Xoo* attack accumulate substantial amounts of ABA from 4 dpi onwards, these levels being significantly higher in susceptible than in resistant plants. Similarly, our quantification of endogenous ABA contents showed that ABA levels strongly increased throughout the course of infection (Table 4.1). In view of these findings, the strong upregulation of ABA-biosynthesis and -responsive genes in control inoculated plants (Figures 4.2A-D; (Liu et al. 2012)), the disease-promoting effect of exogenously administered ABA (Figure 4.1A), and the positive correlation between bacterial growth and pathogen virulence on the one hand, and the amplitude of ABA-responsive gene expression on the other, strongly suggest that virulent *Xoo* may likewise co-opt the rice ABA machinery to promote bacterial growth and cause disease.

In this scenario, the identical bacterial densities observed during the first few days of inoculation in both compatible and incompatible rice-*Xoo* interactions (Figure 4.1B) are suggestive of an ABA-preceding interaction phase during which host and pathogen 'battle' for dominance. Depending on the outcome of this early interaction, *Xoo* strains may or may not be capable of hijacking the rice ABA pathway at late infection to achieve their full virulent potential. Although the molecular mechanisms underlying the early steps of rice-*Xoo* interactions are poorly resolved, recent transcriptome analyses and combined metabolite and hormone profiling increasingly implicate a coordinated range of hormone pathways. For instance, in resistant rice responding to virulent *Xoo*, both JA and ET signaling were found to be strongly activated within one hour after pathogen attack (Grewal et al. 2012), whereas

suppression of auxin and GA signaling seems to occur significantly later, i.e. between 12 hpi and 3 dpi (Yang et al. 2008; Fu et al. 2011). Together with our results, these data therefore suggest that temporally separated transient hormone changes play an important role in configuring the plant's response to *Xoo* attack, with both host and pathogen trying to sequentially engage distinct hormone pathways in defined temporal windows.

ABA suppresses SA-mediated defenses

Over the past decade, a multitude of mechanisms underpinning ABA's broad and divergent impact on plant resistance responses have been identified. Besides interfering with pathogen-induced deposition of callose and modulating production of reactive oxygen species, ABA has been repeatedly shown to influence disease outcomes by interfering with other defense hormones (Asselbergh et al. 2008b; Cao et al. 2011). For instance, antagonistic or synergistic interactions between ABA and JA/ET are well known to play a pivotal role in numerous host-microbe interactions (Anderson et al. 2004; De Vleesschauwer et al. 2010; Bailey et al. 2009; Adie et al. 2007; Lackman et al. 2011). In addition, ABA has been proposed to antagonize SA-mediated signaling to regulate defense responses in tomato and Arabidopsis, where it affects both SA biosynthesis and signaling (Yasuda et al. 2008; De Torres Zabala et al. 2009). In a similar vein, ABA enhances susceptibility of rice to *M. oryzae* by suppressing SA-regulated defenses (Jiang et al. 2010). Interestingly, several lines of evidence suggest that negative ABA-SA crosstalk also underpins the disease-promoting effect of ABA during rice-*Xoo* interactions. First, lesions caused by *Xoo* were more severe on SA-deficient *NahG* plants (Figure 4.6D), whereas topical application of SA or ectopic expression of the SA regulatory genes *OsNPR1* and *OsWRKY13* resulted in enhanced resistance (Figures 4.5D-F), tagging SA as a positive regulator of BLB resistance. Moreover, ABA not only antagonized SA-responsive gene expression in detached leaf assays but also down-regulated the transcription of SA regulatory genes during rice-*Xoo* interactions (Figures 4.4A-C and 4.5A-C) and, accordingly, attenuated SA-inducible pathogen resistance (Figure 4.5D). Finally and consistent with reciprocal antagonism in the direction of SA damping ABA action, we found SA to alleviate ABA-triggered effects on both marker gene expression and pathogen resistance (Figures 4.4A-C and 4.5D). When considered together, these data favor a scenario whereby mutually antagonistic ABA-SA crosstalk plays a central role in shaping the outcome of rice-*Xoo* interactions.

Interestingly, our data also infer that ABA antagonizes the SA signaling pathway downstream of *OsWRKY13* but upstream of *OsNPR1,* as overexpression of *OsNPR1* but not *OsWRKY13* abolished the negative impact of ABA on BLB resistance (Figures 4.5E-F). Potential target

sites for ABA-mediated suppression of SA action include the transcription factors *OsWRKY71* and *OsWRKY24*, both of which function as transcriptional activators of SA signaling and are differentially expressed in response to *OsWRKY13* overexpression and/or ABA treatment (Qiu et al. 2008; Zhang et al. 2009). Alternatively, ABA may activate negative regulators of SA-responsive gene expression that either inhibit or out-compete positive regulators. Recently, Yasuda et al. (2008) identified multiple nodes of confluence between the SA and ABA signaling pathways in Arabidopsis (Yasuda et al. 2008). Exploring whether similar crosstalk mechanisms are operative in rice is a major challenge for future research.

Fluridone-inducible *Xoo* resistance functions independently of SA

In higher plants, endogenous ABA is synthesized predominantly from zeaxanthin, which is an important intermediate in the carotenoid-biosynthesis pathway (Xiong and Zhu 2003). Fluridone is a herbicide that is widely used in ABA-related research because of its ability to block carotenoid synthesis, thus reducing ABA precursor pools. Based on the finding that ABA suppresses resistance to *Xoo* by antagonizing SA defenses and given the strong negative effect of fluridone treatment on bacterial growth and pathogen-induced expression of ABA biosynthesis and response genes (Figures 4.1A-B and 4.3A-C), we initially hypothesized fluridone to enhance resistance to *Xoo* by de-repressing the SA pathway. Surprisingly, however, we failed to observe any significant or reproducible differences in SA-responsive gene expression between control and fluridone-treated plants (Figures 4.6A-C). Moreover, fluridone triggered wild-type levels of resistance in both *OsNPR1* RNAi and SA-deficient *NahG* seedlings, indicating that fluridone-inducible resistance requires neither SA biosynthesis nor SA action (Figures 4.6D-E). Although relatively little is known about the mechanism(s) of fluridone-mediated pathogen resistance, a few studies point to some possibilities. For example, Achuo et al. (2003) reported that micromolar concentrations of fluridone induced resistance of tomato against *Botrytis cinerea* without disturbing the plant ABA pool (Achuo et al. 2003). This result could be explained by assuming that fluridone caused some sort of physiological stress, the response to which resulted in disease resistance. Supporting this hypothesis, fluridone and norflurazone, another inhibitor of ABA biosynthesis, have been shown before to provoke physiological stress in plants through photobleaching of chlorophyll, a phenomenon also observed in this study (Figure 4.1C) (Taylor et al. 2000). Considering the strong impact of abiotic stress factors on plant immunity and the complex interplay between biotic and abiotic stress-response signaling pathways (Fujita et al. 2006; Sharma et al. 2013), it is not unlikely that stress due to mild doses of photobleaching fluridone should result in disease resistance. Therefore, we propose that

fluridone-mediated resistance to *Xoo* does not derive primarily from lowering ABA content and resultant activation of SA-mediated defenses, but rather is due to induction of non-specific physiological stress.

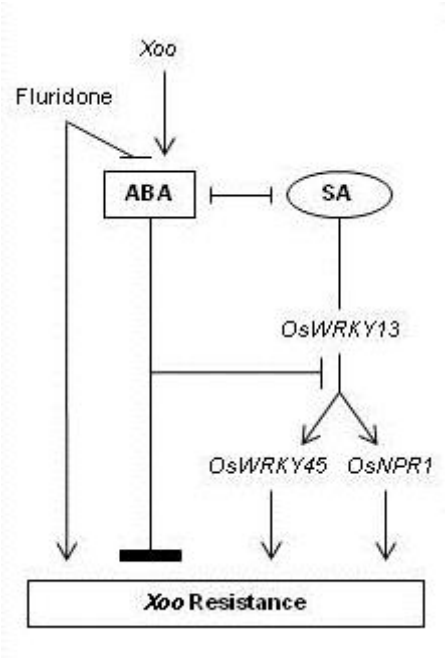

Figure 4.7. Model illustrating how dynamic interplay between ABA and SA molds innate immunity of rice against the BLB pathogen *Xoo*. Sharp arrows represent stimulatory effects, blunt arrows depict antagonistic interactions.

Conclusions

In conclusion, our results favor a model whereby ABA and its interaction with the SA pathway play central roles in orchestrating immunity of rice against the BLB pathogen *Xoo* (Figure 4.7). We propose that ABA acts as a virulence factor for *Xoo* by antagonizing effectual SA-mediated defenses downstream of the master regulator *OsWRKY13* but upstream of *OsNPR1*. In contrast, application of the ABA-lowering herbicide fluridone was found to trigger an SA-independent type of resistance. While bidirectional SA-ABA crosstalk may provide rice with a powerful potential to tailor its immune response to different types of attackers, our results suggest that virulent *Xoo* bacteria have evolved sophisticated strategies to manipulate ABA-SA interplay for their own benefit, redirecting the host immune response in favour of disease.

Materials and Methods

Plant materials and growth conditions

Seeds of the *OsWRKY13*-OX (Qiu et al. 2008), the *OsNPR1*-OX and the *OsNPR1* RNAi transgenics (Yuan et al. 2007) and their respective wild-type lines Mudanjiang and Taipei were kindly provided by Dr. Wang (Huazhong Agricultural University, China) and Dr. He (Shanghai Institute for Biological Sciences, China), respectively. Rice *NahG* (Yang et al. 2004) and *OsMPK5* RNAi (Xiong and Yang 2003) seeds, and their parental line, *japonica* cultivar Nipponbare, were kind gifts from Dr. Yinong Yang (Pennsylvania State University, USA). *Indica* lines IRBB3 and IRBB13 were obtained from the International Rice Research Institute (courtesy of Casiana Vera-Cruz).

Unless stated otherwise, rice plants were grown in a hydroponic gnotobiotic system. Briefly, rice seeds were surface sterilized by agitation in 2 % sodium hypochlorite for 20 min, rinsed three times with sterile demineralized water, and germinated for 5 days at 28 ºC on wet filter paper. Germinated seedlings were first sown in sterilized vermiculite supplemented with half-strength Hoagland solution. Two weeks later, the plants (3-leaf stage) were transferred to plastic containers containing modified Hoagland solution and grown for another three weeks under growth chamber conditions (28 °C, relative humidity: 60%, 12/12 light regimen). For seed multiplication, plants were propagated in the greenhouse (30 ± 4ºC and 16 h photoperiod) and fertilized with 0.5% ammonium sulphate every two weeks until flowering.

Pathogen culture and inoculation assays

Xanthomonas oryzae pv. *oryzae* strain PXO99 (Philippine race 6) (Song et al. 1995) was routinely grown on Sucrose Peptone Agar (SPA) medium at 28 °C. For inoculation experiments, single colonies were transferred to liquid SP medium and grown for 48 h at 28 °C. Plants were inoculated when 6 weeks old by clipping the fifth and sixth stage leaves with scissors dipped in a solution of *Xoo* cells in water (1×10^9 CFU.mL^{-1}). Inoculated plants were kept in a dew chamber (≥ 92% relative humidity; 28 ± 2 ºC) for 24 h and thereafter transferred to greenhouse conditions for disease development. Fourteen days after inoculation, disease severity was assessed by measuring the length of the water-soaked lesions. For bacterial growth analysis, inoculated leaves from three plants were pooled, ground up thoroughly using mortar and pestle and resuspended in 5 to 10 ml water. The leaf suspensions were diluted accordingly and plated on SPA. Plates were incubated at 28 °C and colonies were counted within 2-3 days.

Chemical treatments

Stock solutions of SA (Sigma, Bornem, Belgium) were prepared directly in water, whereas fluridone (Fluka, Bornem, Belgium) and ABA (Duchefa, Schaarbeek, Belgium) were first dissolved in a few drops of methanol and ethanol, respectively. Equivalent volumes of both solvents were added to separate control treatments to ensure they did not interfere with the experiments. Fluridone was applied 6 days before *Xoo* inoculation by adding the compound to the modified Hoagland solution at a concentration of 0.4 µM. ABA and SA, on the other hand, were diluted in 0.02% (v/v) Tween 20 and applied as a foliar spray 72 h before inoculation. Control plants were sprayed evenly with 0.02% (v/v) Tween 20 only. For crosstalk experiments, fresh leaves from 6-week old rice seedlings were detached, cut into 3 cm pieces and subsequently incubated in the indicated hormone solutions for 8 h at 28 ºC. Leaf pieces from 13 plants were pooled and distributed randomly across the different treatments.

RNA extraction and quantitative RT-PCR

Total leaf RNA was extracted using TRIZOL reagent (Invitrogen) and subsequently treated with Turbo DNase (Ambion) to remove genomic DNA contamination. First-strand cDNA was synthesized from 2 µg of total RNA using Multiscribe reverse transcriptase (Applied Biosystems) and random primers following the manufacturer's instructions. Quantitative PCR amplifications were conducted in optical 96-well plates with the Mx3005P real-time PCR detection system (Stratagene), using Sybr Green master mix (Fermentas) to monitor dsDNA synthesis. The expression of each gene was assayed in duplicate in a total volume of 25 µL including a passive reference dye (ROX) according to the manufacturer's instructions (Fermentas). The thermal profile used consisted of an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 59°C for 30 s, and 72°C for 30 s. To verify amplification of one specific target cDNA, a melting-curve analysis was included according to the thermal profile suggested by the manufacturer (Stratagene). The amount of plant RNA in each sample was normalized using *eEF1α* (Eukaryotic elongation factor 1-alpha) as internal control (Jain et al. 2006). Nucleotide sequences of all primers used are listed in Supplemental Table S1. Group-wise comparison and statistical analysis of relative expression results was performed using Relative Expression Software Tool (*REST©)* (Kauffman et al. 1973).

Quantification of ABA

6-week old T65 seedlings were inoculated with PXO99 or sterilized water, subsequently 3 cm leaf blades starting from the inoculation site were sampled at 0, 1, 2, 4, 8 days post inoculation (DPI). These leaf samples were crushed into fine power using the Tissuelyser (Qiagen, Hilde, Germany) and kept in -80 °C until use. The hormone measurement was carried out using the ultra-performance liquid chromatography (UPLC) tandem mass spectrometry (AQITY UPLC System/Quattro Ultima Pt; Waters, Milford, MA, USA) with an ODS column (AQITY UPLC BEH C18, 1.7 µm, 2.1 by 100mm; Waters, Milford, MA, USA). For more details about sample preparation and UPLC protocol, see this paper (Kojima et al. 2009).

Table 4.2. Sequences of qRT-PCR primers used in this study

Gene name	Locus	Forward primer	Reverse primer
eEF1a	LOC_Os03g08020.1	TTTCACTCTTGGTGTGAAGCAGAT	GACTTCCTTCACGATTTCATCGTAA
OsNCED3	LOC_Os03g44380.1	TACGGCTTCCACGGCACGTTC	AGAAACGTGGAGGTGTTCGATCG
OsNCED4	LOC_Os07g05940.1	ATTGCACGGCACCTTCATTG	ACCGCTAAACTATTTCAACTCCCT
OsLip9	LOC_Os02g44870.1	CGGCGGCCTCTTCGAGACAAC	TGCCAGATTGCCAGCCCGTC
OsRab16	LOC_Os01g50700.1	CACGAGTTCAGGGATCTAGGC	AGTTGTCCATCCTCTCAAGCAA
OsNPR1	LOC_Os01g09800.1	CACGCCTAAGCCTCGGATTA	TCAGTGAGCAGCATCCTGACTAG
OsWRKY45	LOC_Os05g25770.1	GGACGCAGCAATCGTCCGGG	CGGAAGTAGGCCTTTGGGTGC
OsWRKY13	LOC_Os01g54600.1	AGCTGCCACGCGAGCAAGTC	GTCCGTCAGCCACCGGCTCAG

Acknowledgements

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Chapter 5

Cytokinin attenuates rice immunity against the bacterial leaf blight pathogen *Xanthomonas oryzae* pv. *oryzae* by activating Target of Rapamycin signaling

Jing Xu, Mikiko Sodeoka, Hitoshi Sakakibara, Monica Höfte and David De Vleesschauwer

In preparation

Abstract

Cytokinins (CKs) are a group of adenine-derived plant hormones that orchestrate myriad growth and developmental processes, including cell division, leaf senescence and source-sink relationships. Although CKs have recently also been implicated in plant-pathogen interactions, little is known about the underlying mechanisms. Here, we have studied the role and dynamics of CKs during the interaction between rice and the bacterial leaf blight pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Through exogenous hormone applications, time-resolved hormone measurements and integrative analyses with CK-modified plants, we found that CKs antagonize basal immune responses against *Xoo*, thereby functioning as a virulence factor for the bacterium. Contrary to recent findings in Arabidopsis, this CK-induced susceptibility functions independently of the classic immune hormone salicylic acid. In contrast, our findings support a model whereby CKs suppress *Xoo* resistance through activation of a plant growth-promoting pathway controlled by the master regulatory protein Target of Rapamycin (TOR). We hypothesize that CK-induced TOR activation disrupts the plant's balance between growth and defense in favor of growth, thereby reducing the amount of resources available for the plant to mount an effective immune responses. Together these findings offer novel insighs into the multifaceted role of CKs in plant stress signaling and underscore the importance of growth-defense trade-offs in molding pathological outcomes.

Introduction

In the absence of adaptive immunity displayed by animals, plants have evolved a plethora of constitutive and inducible defense responses to defend themselves against pathogen attack. Many of these defenses rely on elaborate signaling networks regulated by plant hormones. Upon pathogen attack, plants produce a complex blend of hormones and thereby coordinate downstream defense responses. Salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are the archetypal defense hormones and their importance in the hard wiring of the plant defense signaling circuitry is well established (Pieterse et al. 2012). Other phytohormones, such as abscisic acid (ABA), gibberellins (GAs), auxins, brassinosteroids (BRs) and cytokinins (CKs) are better known for their roles in abiotic stress tolerance or plant growth or development and only recently emerged as important regulators of plant immunity (Robert-Seilaniantz et al., 2011; De Vleesschauwer et al. 2013; De Bruyne et al., 2014).

CKs are a group of N^6-substituted adenine-derived hormones that regulate myriad growth and developmental processes, including cell division, apical dominance, leaf senescence, lateral root formation, and nutritional signaling (Argueso et al. 2009). Naturally occurring CKs

are divided into two groups based on their side chain: those with isoprene-derived side chains, which are predominant in plants; and those with aromatic side chains (Mok and Mok, 2001). In plants, biosynthesis of CKs is controlled by two classes of isopentenyltransferases (IPT). ATP and ADP IPTs are responsible for isopentenyl adenine (iP) and *trans*-type zeatin (tZ) synthesis, whereas tRNA IPTs catalyze *cis*-type zeatin (cZ) synthesis (Miyawaki et al., 2006). In addition to the regulation of its biosynthesis, steady-state levels of active CKs are also determined via conjugation to sugar moieties and degradation by cytokinin oxidases/dehydrogenases (Sakakibara 2006).

According to current concepts, bioactive CKs are perceived and their signals transduced via a multistep phosphorelay that shares homology to the two-component systems (TCS) with bacteria sense and respond to environmental stimuli. A typical CK TCS consists of a hybrid sensor histidine (His) kinase (HK), His-containing phosphotransfer proteins (HP) and a separate response regulator (RR) (Argueso et al. 2009). RRs are classified into three groups (type A, B and C) based on structural features and sequence (Schaller et al. 2008). Type-A RRs are rapidly induced in response to exogenous CKs and act as negative regulators of CK signaling, while phoshorylated type-B RRs serve as transcriptional activators, resulting in the rapid induction of CK-dependent target genes, including type-A RRs (Ishida et al. 2008; Jain et al. 2006). The type-C RRs are structurally similar to type-A RRs but are clearly distinct based on phylogenetic analysis (Schaller et al. 2008; Pils and Heyl 2009).

In addition to their pivotal role in plant growth and development, CKs are also increasingly implicated in plant-pathogen interactions. Increased CK content is classically associated with disease symptoms and pathogen-induced morphological disorders such as galls, tumors and the formation of “green islands” (Giron et al. 2013; Grant and Jones, 2009; Choi et al., 2011). Moreover, many fungal and bacterial pathogens can produce CKs themselves, suggesting a negative role for CK in plant immunity. In line with this notion, Siemens et al. (2006) reported that overexpression of *CKX* in Arabidopsis results in enhanced resistance against the club root pathogen *Plasmodiophora brassicae*, which causes aberrant root growth (Siemens et al. 2006).

Nonetheless, CK can also positively regulate plant defenses. For example, application of CK results in decreased replication of White Clover Mosaic Potexvirus and defense gene expression in bean plants (Li et al. 1999). In Arabidopsis, CKs modulate SA signaling, thereby enhancing resistance to the hemibiotrophic bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) and the biotrophic oomycete *Hyaloperonospora arabidopsidis* (Choi et al. 2010; Argueso et al. 2012; Naseem et al. 2012). However, CKs are also implicated in resistance to necrotrophic pathogens. Thus, transgenic Arabidopsis with

increased CK levels exhibited enhanced resistance to *Alternaria brassicicola*, while delayed leaf senescence resulting from expression of a pathogen-inducible *ipt* gene contributed to resistance of tomato against *Botrytis cinerea* (Choi et al., 2010; Swartzberg et al., 2008).

To date, research aimed at elucidating the defense-associated roles of CK has tended to focus on the use of dicot model plants. In contrast, very little is known about the role of CK in monocots such as rice (*Oryza sativa* L.). Recently, Jiang et al. (2013) reported that CK functions as a two-faced player in defense of rice against the hemibiotrophic leaf blast fungus *Magnaporthe oryzae* (*Mo*), activating SA-mediated plant defenses on the one hand, and facilitating *Mo* infection by enhancing the sink strength of infected tissues, on the other (Jiang et al. 2013). However, whether CK also affects rice defense to other pathogens remains to be elucidated. Aiming to advance our knowledge on the immune-regulatory role of CK, we have explored the function and dynamics of CK in rice defense against *Xanthomonas oryz*ae pv. *oryzae* (*Xoo*), causal organism of the devastating rice leaf blight disease (BLB). By combining exogenous hormone applications, time-resolved hormone measurements and bioassays with CK-modified mutant plants, we found that CK acts as a negative regulator of *Xoo* resistance. Analysis of the underlying mechanisms revealed that this CK-induced disease susceptibility is independent of either SA or auxin signaling. Instead, our findings favor a scenario whereby CK antagonizes basal host immunity by tilting the growth-versus-defense balance towards growth through activation of the ancient growth-regulatory protein Target of Rapamycin.

Results

CK plays a negative role in the defense of rice against *Xoo*

In a first attempt to study the role of CK in resistance of rice against *Xoo*, we examined the effect of exogenously administered kinetin, a naturally occurring aromatic CK, on BLB development. To this end, 6-week old IRBB3 plants were sprayed until run-off with increasing concentrations of kinetin and, three days later, inoculated with virulent *Xoo* strain PXO99. Disease severity was routinely evaluated at 14 days post inoculation (dpi) by measuring the length of the water-soaked lesions characteristic of leaf blight disease. As shown in Figure 5.1A, both low (10 nM and 100 nM) and high concentrations of kinetin (50 µM) significantly increased BLB severity, causing an approximate 40% increase in lesion length relative to non-treated controls. Our previous work showed that *Xoo* growth in rice leaves correlates well with lesion development (Xu et al. 2013). Hence, we also tested the effect of 50 uM kinetin on *Xoo* growth *in vivo*. At 12 dpi, PXO99 titers reached approximately 2.5 x 10^9

CFU/leaf in kinetin-pretreated IRBB3, a greater than 10-fold increase compared to non-treated controls (Figure 5.1B).

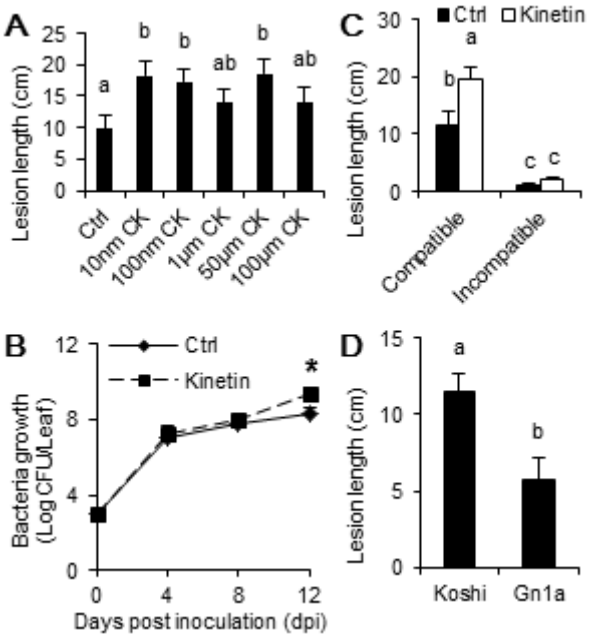

Figure 5.1. Cytokinin (CK) promotes bacterial leaf blight (BLB) development in rice. (A). Six-week old susceptible IRBB3 seedlings were pretreated with increasing concentrations of kinetin, a naturally occurring CK, 3 days before inoculation with virulent *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Disease was evaluated at 14 days post inoculation (dpi) by measuring the length of water-soaked like lesions. Data presented are means ± SE of at least 10 plants. Different letters indicate statistically significant differences (Mann-Whitney: $n \geq 20$; $\alpha = 0.05$). (B). Effect of 50 μM kinetin on PXO99 titers in IRBB3. Data presented are means ± SE of 6 biological replicates from two independent experiments. Asterisks indicate statistically significant differences (Mann-Whitney: $n = 6$; $\alpha = 0.05$). (C). Effect of 50 μM kinetin on BLB development in IRBB3 (compatible) and resistant IRBB13 (incompatible) plants. Data presented are means ± SE of at least 10 plants. Different letters indicate statistically significant differences (Mann-Whitney: $n \geq 20$; $\alpha = 0.05$). (D). Comparison of BLB development in *Gn1a* plants, which contain low levels of bioactive CK, and the wild type (WT) Koshihikari. Data presented are means ± SE of at least 5 plants. Different letters indicate statistically significant differences (Mann-Whitney: $n \geq 10$; $\alpha = 0.05$). Another independent experiment showed similar results.

To assess whether CK influences basal and/or effector-triggered immunity, we next compared the effects of kinetin pretreatment on BLB development in compatible and incompatible rice-*Xoo* interactions. Seedlings of rice lines IRBB3 and the resistant cultivar IRBB13 harboring the recessive resistance gene *xa13* were pretreated with 50 μM kinetin and then inoculated with PXO99. As shown in Figure 5.1C, kinetin pretreatment almost doubled the lesion length in IRBB3 while it was largely ineffective in IRBB13, suggesting that CK may specifically attenuate basal defenses in rice. To test this hypothesis, we performed a set of bioassays with *Gn1a*, a near-isogenic rice line displaying reduced CK activity due to

over-accumulation of conjugated CK bases (Ashikari et al. 2005), and its wild-type (WT) Koshihikari. Corroborating the disease-promoting effect of exogenous kinetin, *Gn1a* was significantly more resistant to PXO99 infection, showing an almost 50% reduction in lesion length compared to inoculated WT plants (Figure 5.1D). Together these data strongly suggest that CK suppresses basal rice immunity to *Xoo* and, hence, acts as a negative regulator of BLB resistance.

Bioactive CKs accumulate in rice leaves after infection with *Xoo*

Over the past decades, several plant pathogenic organisms have been shown to produce CK themselves and/or alter CK biosynthesis in plants (Giron et al. 2013; Spíchal, 2012). To determine the impact of *Xoo* infection on the CK signature of rice, we monitored the CK content of mock- and PXO99-infected plants at various times post inoculation using highly sensitive ultra-performance liquid chromatography electrospray tandem mass spectrometry (Kojima et al. 2009). As shown in Table 5.1, no major and/or significant differences between treatments could be observed at either 1, 2 or 4 dpi, except for a rapid and prolonged increase in the levels of cZ. At 8 dpi, however, pathogen-inoculated leaves exhibited a strong rise in the levels of the CK nucleobases iP, tZ, cZ and DZ (dihydrozeatin) and their corresponding ribosides and nucleotides (tZR, tZRPs, cZR, cZRPs, DZRPs, iPR and iPRPs). While free base CK species are generally more active than the riboside forms, CKs can also be reversibly modified by conjugation of glucose to the hydroxyl group of the side chain; the corresponding *O*-glucosides being less active than the unglycosylated forms (Sakakibara 2006). In addition, CKs can be irreversibly inactivated by conjugation to glucose at the N^3, N^7 and N^9 positions of the adenine ring (Mok and Mok 2001). Intriguingly, none of these glycosylated CK species were significantly affected by PXO99 infection, suggesting that *Xoo* induces accumulation of active CKs by increasing *de novo* hormone biosynthesis rather than affecting CK inactivation or degradation processes.

Table 5.1 Concentrations (pmol/g FW) of CKs in T65 leaves after infection with virulent *Xoo* strain PXO99

dpi	0	1		2		4		8	
	Mock	Mock	Infected	Mock	Infected	Mock	Infected	Mock	Infected
tZR	3.27±ND	0.47±ND	0.75±ND	0.63±0.13	0.71±0.2	0.68±0.13	0.74±0.19	1.04±ND	4.27±1.89
tZRPs	4.31±ND	2.11±ND	3.55±ND	1.07±0.2	1.09±0.34	1.95±0.6	1.7±0.04	1.91±ND	12.31±3.51
cZ	2.22±ND	5.82±ND	21.23±ND	2.51±1.24	26.66±11.55	14.09±8.65	17.87±3.08	8.29±ND	24.41±3.16
cZR	1.55±ND	1.6±ND	2.44±ND	1.03±0.12	3.09±1.45	3.14±0.91	3.77±1.41	10.6±ND	35.46±7.45
cZRPs	0.55±ND	0.67±ND	1.95±ND	0.55±0.11	2.4±0.91	1.32±0.67	1.74±0.34	1.56±ND	22.66±11.56
DZ	0.11±ND	0.25±ND	1.16±ND	0.29±ND	1.51±0.5	0.66±0.4	1.12±ND	0.34±ND	2.27±ND
iP	0.56±ND	0.15±ND	0.36±ND	0.19±0.04	1.35±2.01	1.01±0.5	2.87±3.63	0.78±ND	6.6±6.55
iPR	0.51±ND	0.11±ND	0.28±ND	0.1±0.02	0.49±0.5	0.6±0.41	1.13±0.79	1.14±ND	21.07±7.39
iPRPs	5.52±ND	1.75±ND	3.32±ND	2.09±0.31	4.49±4.09	5.76±2.28	11.27±7.63	8.04±ND	40.54±7.35
tZ9G	245.64±ND	104.94±ND	114.2±ND	139.22±33.62	134.51±43.19	126.92±9.89	113.18±48.17	116.45±ND	74.35±10.87
tZOG	4.44±ND	4.66±ND	5.46±ND	4.56±0.55	4.04±0.71	3.83±0.88	4.27±0.26	3.16±ND	2.35±0.59
cZOG	1357.29±ND	1564.81±ND	1691.62±ND	1499.77±70.78	1414.59±204.9	1370.93±92.65	1637.09±165.57	874.68±ND	1002.31±86.83
cZROG	32.31±ND	15.68±ND	19.24±ND	15.47±0.78	19.28±5.7	24.66±3.78	28.7±15.13	32.76±ND	25.29±2.11
cZRPsOG	1.63±ND	1.86±ND	3.57±ND	1.59±0.14	2.73±0.62	2.91±0.94	2.55±0.26	1.85±ND	3.11±0.95
DZ9G	3.32±ND	2.02±ND	1.86±ND	1.79±0.11	1.79±0.32	2.27±0.34	2.13±0.18	2.37±ND	1.57±0.47
iP9G	7.16±ND	4.02±ND	4.62±ND	4.44±2.27	8.12±7.69	8.41±1.78	7.98±1.21	6.32±ND	8.29±3.17

Data presented are mean ± SD of three biological repetitions. tZR, trans zeatin riboside; tZRP, tZR phosphates; cZ, cis zeatin; cZR, cZ riboside; cZRP, cZR phosphates; DZ, dihydrozeatin; iP, N6-(Δ2-isopentenyl) adenine; iPR, iP riboside; iPRP, iPR phosphates; tZ9G, tZ 9-glucoside; tZOG, tZ O-glucoside; cZOG, cZ O-glucoside; cZROG, cZR O-glucoside; cZRPsOG, cZPs O-glucoside; DZ9G, DZ 9-glucoside; iP9G, iP 9-glucoside; ND: not determined because there were only two samples. Other CK species were not detected. dpi: days post inoculation.

CK-induced susceptibility to *Xoo* is independent of SA

In Arabidopsis, CK has been found to influence disease outcomes by positively interacting with the SA signaling pathway (Argueso et al. 2012; Choi et al., 2010). Similarly, Jiang et al. (2013) reported that CK acts in concert with SA to activate pathogenesis-related (PR) defense gene expression in rice (Jiang et al. 2013).

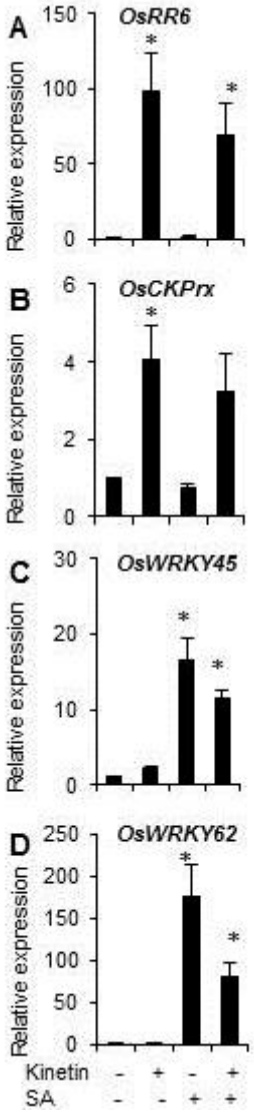

Figure 5.2. Effect of exogenously administered kinetin (50 µM) and/or SA (500 µM) on reciprocal hormone pathways. (A) through (D). qRT-PCR analysis of the expression of the CK-responsive genes *OsRR6* and *OsCKPrx*, and the SA marker genes *OsWRKY45* and *OsWRKY62*. Data are means ± SE of two technical and two biological replicates, each biological replicate representing a pooled sample from 13 individual plants. Asterisks indicate statistically different significance compared control (T-test, n=4, α=0.05).

To further explore the nature and direction of SA-CK crosstalk in rice, we tested the effect of single and combined hormone treatments on the expression of SA and CK marker genes.

For this purpose, 3-cm-long leaf segments of IRBB3 were incubated for 8 h in aqueous solutions containing 50 µM kinetin and/or 500 µM SA and tested for expression of several CK and SA marker genes, including *OsWRKY45*, *OsWRKY62*, *OsRR6* and *OsCKPrx*. Unlike the situation in Arabidopsis where NPR1 controls almost all SA-responsive genes, the SA signaling pathway in rice branches into two sub-pathways controlled by OsWRKY45 and OsNPR1, respectively (Qiu et al. 2008; Tao et al. 2009). *OsWRKY62* is a transcription factor gene located downstream of *OsWRKY45* (Mao et al. 2007), while *OsRR6* is a type-A RR gene that is transcriptionally upregulated in response to CK but negatively feedbacks on downstream CK signaling in a phosphorylation-dependent manner (Sakakibara et al. 2007). Finally, *OsCKPrx* is a CK responsive gene encoding a CK-inducible peroxidase (Sakakibara et al. 2007). As expected, kinetin strongly up-regulated the expression of *OsCKPrx* and *OsRR6*, while SA induced the expression of *OsWRKY45* and *OsWRKY62* (Figures 5.2A-5.2D). Moreover, although kinetin and SA did not affect transcription of the reciprocal marker genes, hormone co-application slightly reduced SA- and CK-responsive gene expression relative to single hormone treatments (Figures 5.2B-5.2D), which is suggestive of weak reciprocal antagonism between the two pathways.

To further invetigate the interaction between CK and SA, we next assessed the effect of exogenously administered kinetin on CK-responsive gene expression in detached leaves of *OsNPR1* RNAi and *OsNPR1* overexpression (*NPR1* OX) plants. As shown in Figures 5.3A, we failed to observe any substantial differences between the *OsNPR1* transformants and corresponding WT plants in either basal or kinetin-inducible expression of *OsRR6*. Similarly, *OsNPR1* RNAi plants also showed WT expression levels of *OsRR2* and *OsCKPrx* irrespective of CK treatment. In contrast, *OsNPR1* overexpression suppressed basal and kinetin-inducible expression of *OsCKPrx* and *OsRR2*, respectively, which raises the prospect of negative crosstalk in the direction of SA damping CK action (Figures 5.3B and 5.3C).

Fairly similar findings were obtained when testing the effect of exogenous kinetin on the seedling development of *OsNPR1* transgenic plants. In these experiments, surface sterilized seeds of *OsNPR1* RNAi, *OsNPR1* OX and WT Taipei were grown for 7 days on GB5 medium supplemented or not with 50 µM kinetin, after which we recorded the length of the shoot and primary roots. As shown in Figure 5.3D and 5.3E, non-treated *OsNPR1* OX seedlings had shorter shoot and roots than *OsNPR1* RNAi and WT plants, which is consistent with previous observations (Personal communication with Dr. Zuhua He). Moreover, in line with the results reported by Tsai et al. (2012), exogenous kinetin treatment significantly inhibited root and shoot elongation in WT seedlings, causing an approximate 50% reduction relative to solvent-treated controls. Similar reductions were observed in *OsNPR1*

OX line while the reduction in root and shoot elongation was slightly greater in the *OsNPR1* RNAi background, further suggesting the occurrence of weak SA-CK antagonism.

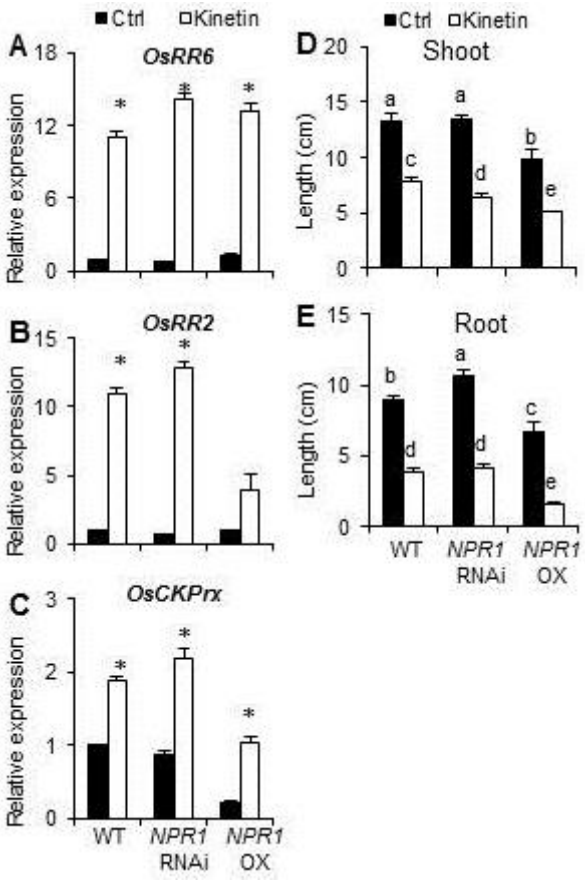

Figure 5.3. Effect of exogenously administered kinetin on CK signaling responses in *OsNPR1* mutants and the WT Taipei. (A) to (C). Expression of the CK-responsive genes *OsRR6*, *OsRR2* and *OsCKPrx* in *OsNPR1* RNAi and *OsNPR1* overexpression (OX) lines and WT plants in response to exogenous kinetin (50 µM). Data are means ± SE of two technical and two biological replicates, each biological replicate representing a pooled sample from 5 individual plants. Asterisks indicate statistically different significance compared to the corresponding control (T-test, n=4, α=0.05). (D) and (E). Seedling development of *OsNPR1* RNAi, *OsNPR1* OX and WT plants in response to exogenous kinetin (50 µM). Data are means ± SE of at least 18 plants from three to four plates. Different letters indicate statistically significant differences (Mann-Whitney: n ≥ 18; α = 0.05).

To test the importance of such putative SA-CK interplay in regulating rice-*Xoo* interactions, we first quantified the effect of exogenously administered NaSA on the level of CK-inducible susceptibility. As expected, NaSA significantly increased resistance compared to inoculated controls, confirming the importance of SA in basal immunity to *Xoo*. However, hormone co-applications revealed that NaSA is unable to suppress the disease-promoting effect of exogenous kinetin (Figure 5.4A). Furthermore, although *OsNPR1* OX plants showed enhanced disease resistance compared to non-treated WT plants, exogenous kinetin treatment proved to be equally effective in both genotypes (Figure 5.4B). Therefore, despite

there being evidence of negative albeit weak SA-CK signal interactions in naïve tissues, these findings suggest that such SA-CK antagonism is of minor importance in shaping rice-*Xoo* interactions.

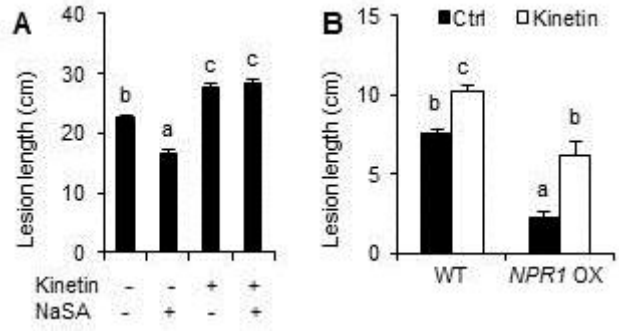

Figure 5.4. CK induces susceptibility to *Xoo* in an SA-independent manner. (A). Effect of 50 µM kinetin and/or 1 mM sodium salicylate (NaSA) on BLB development in susceptible T65 plants infected with PXO99. Data presented are means ± SE of at least 16 plants. Different letters indicate statistically significant differences (Mann-Whitney: $n \geq 32$; $\alpha = 0.05$). (B). Effect of kinetin (50 µM) on BLB development in *OsNPR1* OX and WT plants. Data presented are means ± SE of at least 6 plants. Different letters indicate statistically significant differences (Mann-Whitney: $n \geq 12$; $\alpha = 0.05$).

Auxin and CK interact in a mutually antagonistic manner

Auxin intimately interacts with CK in regulating numerous aspects of plant growth and development. Moreover, exciting new findings indicate that antagonism between the auxin and CK signaling pathways underpins CK-mediated disease resistance in Arabidopsis (Naseem and Dandekar 2012). Therefore, and given the apparent SA-independence of CK-induced susceptibility to *Xoo*, we sought to extend our analysis of the role of CK in *Xoo* pathogenicity by studying the nature and direction of CK-auxin signal interactions in rice. To this purpose, detached leaf segments of five-week-old T65 plants were incubated for 8 h in aqueous solutions containing 50 µM kinetin and/or 100 µM indole acetic acid (IAA), the main auxin in rice, and subsequently tested for expression of several CK and auxin marker genes. As shown in Figures 5.5A to 5.5C, single kinetin treatment significantly activated expression of the CK responsive genes *OsRR6* and *OsRR2* and suppressed expression of the auxin marker gene *OsIAA9*. Moreover, kinetin also antagonized IAA-induced expression of *OsIAA9*, indicating negative crosstalk in the direction of CK suppressing IAA. Yet, consistent with bidirectional CK-IAA antagonism, IAA treatment also suppressed basal expression of *OsRR6* and *OsRR2*. However clear, these data are hard to reconcile with the role of auxin in promoting BLB symptom development (Ding et al. 2008; Fu et al. 2011). Although being preliminary,, it seems unlikely that the negative CK-auxin cross-communication is a major factor contributing to CK-induced *Xoo* susceptibility.

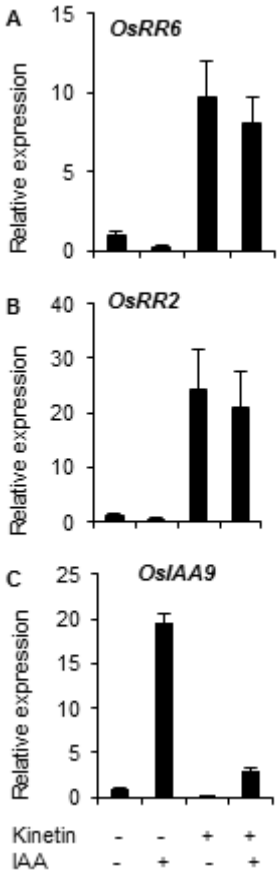

Figure 5.5. Rice CK and auxin pathways display mutual antagonism. T65 leaf segments were incubated in solutions containing 50 µM kinetin and/or 100 µM IAA for 8 h, and subsequently tested for expression of the CK-responsive genes *OsRR6* and *OsRR2* and the auxin-responsive gene *OsIAA9*. Data are means ± SE of two technical replicates from a pooled sample from 13 individual plants.

CK induces *Xoo* susceptibility by activating Target of Rapamycin signaling

Recently, our functional analysis of the ancient growth-regulatory protein Target of Rapamycin (TOR) revealed that the rice TOR protein (OsTOR) negatively controls basal immunity against *Xoo* (De Vleesschauwer et al., unpublished). Acting at the interface of stress, developmental and metabolic signaling, TOR constitutes an evolutionary conserved master switch for activation of eukaryotic cell growth under nutrient-rich conditions (Xiong and Sheen 2014). However, TOR not only coordinates plant growth and development, but also seems to antagonize plant immune responses, thus acting as a key regulatory protein controlling growth-defense dynamics (De Vleesschauwer et al., unpublished). Accumulating evidence also indicates that TOR is intimately associated with plant hormone signaling and is activated by growth-promoting hormones such as GAs (De Vleesschauwer et al.,

unpublished). In view of these findings and aiming to shed further light into the molecular mechanisms by which CK inflicts disease susceptibility, we asked whether CK may suppress basal immunity against *Xoo* by activating the rice TOR signaling pathway.

To this end, we first tested the impact of the TOR-specific inhibitor rapamycin on CK-inducible gene expression in detached leaf assays. Consistent with above-mentioned results (Figure 5.6A), 50 μM kinetin strongly up-regulated expression of *OsRR6*; however, this induction was severely attenuated upon rapamycin treatment (100 μM), suggesting that TOR signaling is indispensable for expression of CK-responsive genes, at least for *OsRR6*. Interestingly, TOR action also seems to be important for CK effects on plant growth and development as revealed by analysis of 7-day-old T65 seedlings grown on GB5 medium supplemented or not with 50 μM kinetin and/or 100 μM rapamycin. Whereas kinetin-treated plants displayed stunted growth and severe inhibition of primary root elongation compared to non-treated control plants, these effects were partially relieved upon co-application with rapamycin (Figures 5.6B and 5.6C). Moreover, single rapamycin treatments induced a peculiar phenotype characterized by a partial loss of root gravitropism and impaired root hair formation, both of which phenotypes were still evident in kinetin/rapamycin co-treatments (Figure 5.6C). These findings suggest that suppression of TOR due to rapamycin treatment leads to failure in activating CK signaling by exogenous kinetin application, which raises a hypothesis that TOR operates downstream of CK and is required for full-blown activation of the CK pathway.

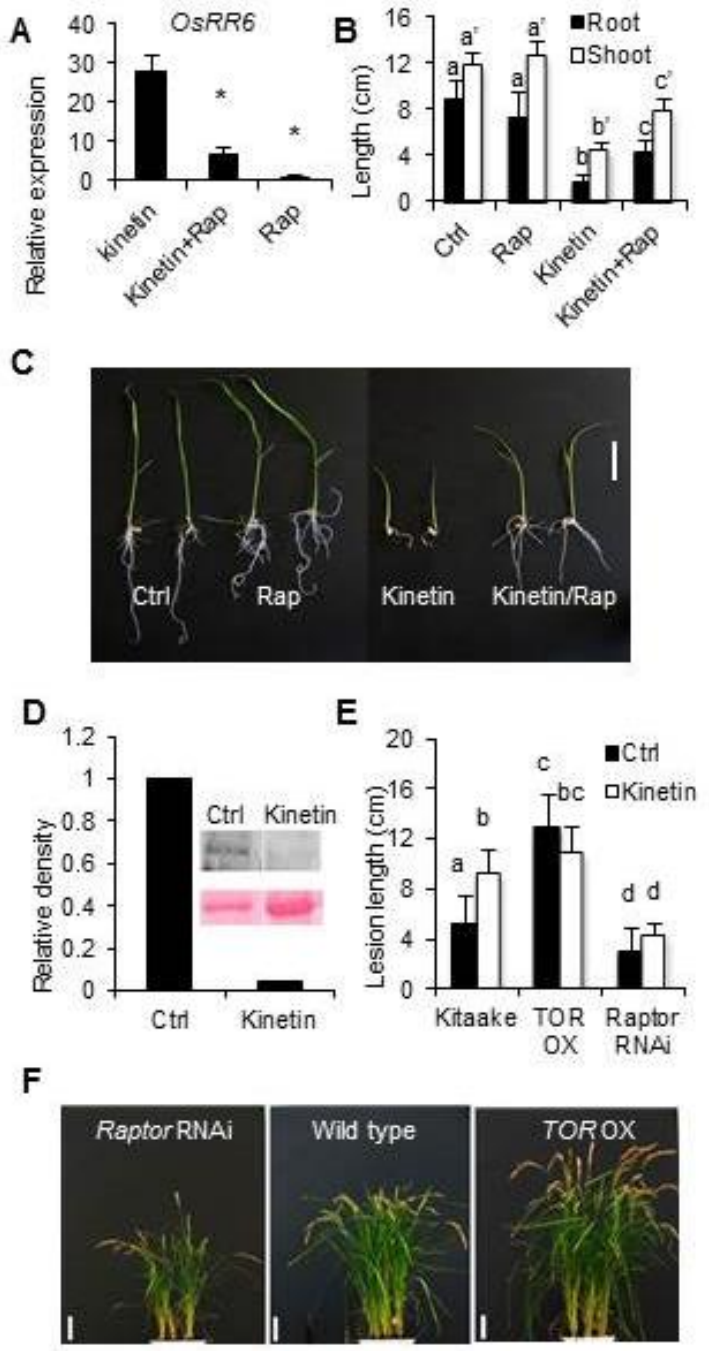

Figure 5.6. CK activates TOR (target of rapamycin) signaling. (A). Effects of kinetin (50 µM) and/or rapamycin (100 µM) on the expression of the CK-responsive gene *OsRR6*. Data are means ± SE of two technical replicates. Asterisks indicate statistically different significance compared to control (T-test, n=4, α=0.05). (B) and (C). Effect of kinetin (50 µM) and/or rapamycin (100 µM; Rap) on seedling development of Kitaake plants. Data presented are means ± SE of 12 plants from two plates. The length of the shoot and primary roots was recorded after 7 days of incubation. Scale bar is 4 cm. (D). Kinetin treatment induces degradation of the TOR antagonist SnRK1. Protein levels were analyzed by western blotting using a polyclonal antibody specific for rice SnRK1 (upper panel). Lower panel shows loading controls (Ponceau staining). Band densities were quantified using ImageJ software. (E) Effect of kinetin pretreatment on BLB development in *TOR* OX, *Raptor* RNAi and WT Kitaake plants. Data are means ± SE of at least 10 plants. Different letters indicate statistically significant differences (Mann-Whitney: n ≥ 10; α = 0.05). (F) Growth phenotype of adult WT Kitaake, *TOR* OX and *Raptor* RNAi plants. Pictures were taken 12 weeks post germination. Scale bar is 10 cm.

In this scenario, one would expect CK to activate TOR signaling. Because there are currently no rice anti-TOR antibodies available, we tested this hypothesis by analyzing the impact of exogenous kinetin treatment on the stability of SnRK1, a well-studied energy sensor kinase that antagonistically interacts with the TOR pathway. Acting as a TOR counterpart by shutting down anabolic reactions and promoting catabolism, TOR and SnRK1 levels are inversely correlated, establishing the amount of SnRK1 as a suitable proxy for TOR activity (Ghillebert et al. 2011; Smeekens et al. 2010). Interestingly, Western blot analyses using polyclonal rice anti-SnRK1 antibodies indicated that 50 μM kinetin strongly reduced steady-state levels of SnRK1, suggesting a positive effect of CK on TOR signaling output (Figure 5.6D).

Having found that CK and TOR behave as collaborative signals, we finally sought to assess the significance of this interaction in *Xoo* pathogenicity by quantifying the level of kinetin-induced susceptibility on several rice transformants with reduced or increased TOR pathway activity. All lines were routinely treated with 50 μM kinetin 3 days prior to challenge with PXO99. Consistent with TOR acting as a negative regulator of *Xoo* resistance, overexpression of TOR improved growth and rendered plants hyper-susceptible to infection, whereas RNAi-mediated silencing of Raptor, an essential member of the TOR protein complex, resulted in reduced growth and enhanced resistance as compared to non-treated WT (Figures 5.6E and 5.6F). Furthermore, despite inducing substantial levels of susceptibility on WT Kitaake plants, kinetin failed to significantly alter the resistance level in the *TOR* OX and *Raptor* RNAi background (Figure 5.6E). In view of abovementioned results, we interpret these data to suggest that CK antagonizes basal immunity at least in part by activating the TOR pathway.

Discussion

In this study, we examined the function and dynamics of the plant hormone cytokinin (CK) in the immune response of rice against the (hemi)biotrophic bacterium *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Through a combination of exogenous hormone applications, multicomponent hormone measurements and bioassays with CK-modified rice plants, we found that CK negatively regulates resistance to *Xoo* and possibly acts as a virulence factor for the bacterium.

As one of the latest growth regulators to be implicated in plant immunity, much remains to be learned about the immune-regulatory role of CK and its position within the signal transduction paths leading to disease and resistance. Nevertheless, a complex picture is emerging from

the limited data available whereby there is no apparent dichotomy between the effectiveness of the CK pathway and the lifestyle or infection strategy of the invading pathogen. Indeed, analyses of various model systems including dicot and monocot plants have revealed both positive and negative effects of CK on disease resistance against (hemi)biotrophic and necrotrophic pathogens (Giron et al. 2013; Choi et al. 2011). Another layer of complexity is added by the sometimes contrasting effects triggered by different types and concentrations of CK. For instance, Argueso et al. (2013) reported that low concentrations of CK (<1 μM benzyl adenine) increased susceptibility of Arabidopsis to the biotrophic oomycete pathogen *H. arabidopsidis*, whereas higher concencentrations (> 10 μM) enhanced resistance. These results echoe previous findings in the wheat-powdery mildew pathosystem, in which a complex dose response curve of pathogen growth was observed in response to exogenous zeatin (Babosha 2009). In rice, high doses of kinetin rendered plants hypersusceptible against the rice blast fungus *Magnaporthe oryzae*, while low levels had little effect on disease susceptibility. In contrast, we found enhanced susceptibility against *Xoo* in response to both low and high kinetin concentrations (Figure 5.1A), suggesting that CK negatively regulates *Xoo* resistance in a concentration-independent manner. Together these apparently conflicting reports illustrate that CKs, much like other growth regulators such as GAs and BRs (De Bruyne et al. 2014), function as multifaceted regulators of plant immunity, the effect of which may depend not only on the plant species and overall infection biology of the invading pathogen, but also on specialized features of each interaction.

Interestingly, contrary to its clear effect on IRBB3 plants that are susceptible to *Xoo* strain PXO99, CK failed to enhance susceptibility on either IRBB13 plants carrying the recessive *R* gene *xa13* or transgenic Kitaake plants harboring the dominant *R* gene *Xa21* (Figure 5.1C; data not shown). These findings suggest that CK predominantly affects basal defenses against *Xoo*, rather than interfering with *R* protein-mediated immunity. However, considering that CKs are well-known to produce differential effects on *R* gene-triggered programmed cell death (Walters et al. 2008), it cannot be ruled out that the effect of CK is dependent upon the type of resistance gene involved, as was previously observed in rice-blast interactions (Jiang et al. 2013). Therefore, additional bioassays using multiple *Xoo* strains and rice lines carrying other types of *R* genes should be carried out to unequivocally delineate the role, if any, of CKs in *R* gene-triggered immune responses.

In line with its effect in promoting disease development, exogenous CK treatment also increased *Xoo* growth in rice leaves (Figure 5.1B). The mechanisms underlying this gain-of-susceptibility phenotype may involve several physiological processes that are regulated by CKs, such as sink-source relationships, delay of senescence and/or nutrient acquisition, many of which likely affect the ability of pathogens to grow optimally (Giron et al. 2013).

Many pathogens produce and secrete CKs, thereby triggering physiological and developmental alterations that suit their nutritional requirements (Walters et al. 2008). Particularly noteworthy in this respect is a recent report showing that rice plants expressing a bacterial *IPT* gene under control of a pathogen-inducible promoter accumulate high amounts of sucrose and starch in flag leaves (Peleg et al. 2011). In this context, the ability of nM concentrations of kinetin to induce BLB susceptibility (Figure 5.1A) suggests that *Xoo*-derived CKs may assist bacterial growth *in planta*. Analysis of the *Xoo* genome revealed the presence of a single ADP/ATP-dependent isopentenyl transferase gene predicted to synthesize iP- and tZ-type CKs. Moreover, several genes encoding tRNA isopentenyl transferases are present in the *Xoo* genome, as they are in most genomes. Although tRNA-derived CKs are considered to be of minor importance in plant physiology, some plant species such as maize and rice contain substantial amounts of cZ-type CKs which have been recently suggested to maintain a minimal level of CK responses accompanied by growth-limiting conditions (Sakakibara 2006; Gajdosová et al. 2011). Exploring the ability of *Xoo* to synthesize CKs and delineating the importance of such microbial CK production in bacterial pathogenicity should be interesting topics for future research.

The past few years have provided many fascinating insights into the ingenious ways by which pathogens manipulate or subvert host hormone pathways for their benefit. One of the common tactics employed by successful pathogens is to trick the plant into activating a phytohormone pathway that confers susceptibility. For example, our previous work showed that virulent *Xoo* boosts *de novo* ABA synthesis in rice leaves (Chapter 4), while Ding et al. (2008) reported a similar effect of *Xoo* infection on auxin synthesis. In common with these findings and corroborating the increase in CK synthesis seen in response to the rice blast fungus *Mo* (Jiang et al. 2013), *Xoo*-infected leaves also showed a strong rise in the level of various bioactive CK species and their corresponding precursors (Table 5.1). Together with the disease-promoting effect of exogenously administered kinetin and the heigthened resistance of CK-deficient *Gn1a* plants (Figures 5.1A and 5.1D), these findings strongly suggest that *Xoo* usurps the rice CK pathway to induce a state of susceptibility. Hann et al. (2014) recently showed that HopQ1, a type III effector from *Pst*, activates CK signaling and interferes with plant innate immunity in Arabidopsis. Since HopQ1 seems to be conserved in other pathogenic bacteria including *Ralstonia* and *Xanthomonas* (Hann et al. 2014), it will be interesting to test whether these HopQ1 homologs perform functionally similar roles in targeting host CK signaling.

In Arabidopsis, CK synergistically interacts with SA to promote *PR1* expression in a process that involves the CK-activated transcription factor ARR2 and the SA response factor TGA3 (Choi et al., 2011). Similarly, Jiang et al. (2013) reported that co-treatment of rice leaf blades

with CKs and SA, but not with either one alone, activates expression of the defense genes *OsPR1b* and *OsPBZ1*. These findings suggest that CK and SA act as collaborative signals in the rice defense network. However, we repeatedly failed to reproduce these results, which might be explained by differences in plant growth conditions and/or the rice cultivar used for these assays (data not shown). Moreover, our gene expression results pointed towards slight antagonism between SA and CK (Figures. 5.2 and 5.3), suggesting that the nature of the CK-SA signal interaction in regulating gene expression depends on the particular type of gene. Nonetheless, our results also suggest that SA-CK signal interactions are of minor importance in molding rice-*Xoo* interactions (Figure 5.4). Consistent with this, CK has been shown before to modulate disease resistance independent of SA. In tobacco, for instance, CK induced resistance against *P. syringae pv. tabaci* by enhancing production of two antimicrobial phytoalexins, scopoletin and capsidiol (Grosskinsky et al. 2011). CK-induced production of phytoalexins has also been observed in rice and some recent studies have demonstrated a positive correlation between phytoalexin accumulation and rice resistance to *Xoo* (Ke et al. 2014; Li et al. 2012; Liu et al. 2012; Ko et al., 2010). These findings may seem conflicting with the negative role of CK in *Xoo* resistance as proposed in this study. However, as seen in other pathosystems, it is not inconceivable that CK participates in multiple defense-associated processes with potentially contrasting outcomes, the CK-inflicted susceptibility to *Xoo* being the net effect of these combined actions.

Plant defense to pathogen attack is commonly associated with a profound reconfiguration of the plant primary and secondary metabolism. Since primary and secondary metabolic pathways use common precursors, allocation strategies are constrained by a restricted pool of available nutrients, explaining the innate trade-off between growth and defense in plants (Huot et al. 2014). Interestingly, several lines of evidence suggest that *Xoo* may usurp CK to manipulate the plant's growth versus defense conflict to its own benefit by activating a central growth-promoting pathway controlled by the master regulatory protein Target of Rapamycin. First, application of the TOR-specific inhibitor rapamycin attenuated the effect of CK in both gene expression experiments and plant growth assays (Figures 5.6A and 5.6B), establishing TOR as an key signal transducer in the CK pathway. Second, application of CK induced degradation of the TOR counterpart SnRK1 (Figure 5.6D), which is indicative of CK promoting TOR activity. Third, overexpression of TOR not only led to a substantial increase in plant biomass, but also rendered plants hypersusceptible to *Xoo* infection, whereas silencing of Raptor, a TOR interaction partner, resulted in enhanced resistance (Figure 6E). Together with the inability of kinetin to cause an additive effect on susceptibility on TOR OX plants, these findings strongly suggest that activation of TOR signaling is an important factor contributing to CK-induced *Xoo* susceptibility.

Although the mechanism of TOR action in plant immunity against *Xoo* is still poorly understood, a few studies point to some possibilities. For instance, besides co-operating with CK, TOR has also been shown to antagonize JA, the effect of which in triggering resistance to *Xoo* is well described (De Vleesschauwer et al., unpublished; De Vleesschauwer et al., 2013). Moreover, given the emerging roles of plant TOR in carbon partitioning and nutrient signaling (Xiong and Sheen 2014), one may speculate that pathogen-induced activation of TOR increases the sink strength of colonized host tissues, thus causing nutrient mobilization towards the infection site. In support of this hypothesis, it was previously shown that SnRK1 allows tobacco to tolerate herbivory by rapidly allocating sugars to roots (Schwachtje et al. 2006). Whichever mechanism operative, manipulating host TOR signaling and hijacking TOR-SnRK1 crosstalk mechanisms likely represents a powerful virulence strategy given the vital role of TOR and SnRK1 in coordinating and integrating myriad cellular, developmental, and physiological processes (Smeekens et al. 2010; Xiong and Sheen 2014).

Conclusion

In summary, we have shown that both exogenously administered and endogenous CKs antagonize resistance against the rice leaf blight pathogen *Xoo*. Moreover, our results favor a scenario whereby the CK-induced susceptibility is independent of SA and auxins, but relies on suppression of basal defense responses through activation of the ancient master regulatory protein Target of Rapamycin. Considering the central importance of TOR in integrating nutrient and energy signaling with a wide variety of hormone and environmental signals, further elucidation of the precise mechanisms via which *Xoo* taps into the TOR network will not only provide important clues as to how plants balance growth and immunity and promote survival under adverse conditions, but also be instrumental in the development of improved crops that are better able to withstand multiple stresses.

Materials and methods

Plant materials and growth conditions

Two *indica* rice cultivars IRBB3 and IRBB13, and a *japonica* cultivar Taichung 65 (T65) were routinely used in this study. Seeds of *japonica* cultivar Koshihikara and Gn1a, a near-isogenic line with a reduced overall CK activity (Ashikari et al. 2005), were kindly provided by Dr. Makoto Matsuoka (Riken, Japon). Seeds of *OsNPR1* RNAi and *NPR1* overexpression

lines and their wild type Taipei (Yuan et al. 2007) were a kind gift of Dr. Zuhua He (Shanghai Institute for Biological Science, China). Rice seeds were surface sterilized in 2% sodium hypochlorite for 20 min, rinsed with sterilized water for 3-5 times, and germinated for 5 days at 28 °C. After germination, the seedlings were transplanted in soil and grown for 4.5 weeks under greenhouse conditions (30 ± 4°C, relative humidity: 60%, 16/8 light regimen). Plants were weekly fertilized with 0.5% ammonium sulphate and 0.5% iron sulphate. For seed multiplication, plants were grown under the same conditions and fertilized until flowering.

Generation of Transgenic Plants

Full-length cDNA clones of TOR and Raptor were amplified with gene-specific primers and cloned into the pENTR/D vector. Individual constructs were recombined into the Ubi/NC1300 overexpression vector and the pANDA RNAi vector, respectively. All constructs were transformed into Kitaake rice (*japonica*) using routine *Agrobacterium*-mediated transformation protocols (Seo et al. 2011). All assays were performed with homozygous T2 lines which showed the typical stunted phenotype.

Chemical treatments

Stock solutions of kinetin (Fluka), SA (Sigma) and sodium salicylate (NaSA) (Sigma) were prepared in 0.1 N NaOH, and distilled water, respectively, filter sterilized and kept at -20 °C. Equivalent volumes of both solvents were added to separate control treatments. For foliar spray treatments, hormone solutions were diluted in 0.02% Tween 20 and applied till run-off 3 days before *Xoo* inoculation. In case of CK application, control plants were sprayed evenly with 0.02% Tween 20 containing equivalent volumes of NaOH. For crosstalk experiments, the two youngest fully developed leaves from 6-week-old rice seedlings were cut into 3 cm pieces, floated overnight on sterilized distilled water to eliminate residual wound responses, and subsequently incubated in the indicated hormone/rapamycin solutions for 8 h at 28°C. Leaf pieces from at least 5 plants were pooled and distributed randomly across the different treatments.

Hormone-response bioassays in young seedlings

Following sterilization as described above, dehulled seeds were pre-germinated on wet sterilized filter paper in sealed Petri dishes at 28°C. Three days post imbibition, germinated seeds were transferred to vertical Petri dishes containing Gamborg B5 medium supplemented with hormones and/or rapamycin at the specified concentrations. Plates were incubated for one week under standard growth chamber conditions (28 °C, relative humidity: 60%, 12/12 light regimen), and evaluated by measuring the length of the primary root and and shoots. At least three plates, each containing six plants, were used per treatment.

Pathogen culture and inoculation assays

Xanthomonas oryzae pv. *oryzae* strain PXO99 (Philippine race 6) was routinely used throughout this work (Song et al. 1995). Inoculum production, plant inoculation and disease evaluation and bacterial growth analysis were performed as described in Chapter 4.

RNA isolation and qRT-PCR

RNA extraction, DNase treatment, cDNA synthesis and qPCR analysis were conducted as described in Chapter 4. The sequences of QPCR primers used in this work are listed in Table 5.2.

Western-Blot analysis

Extraction of total protein and western blotting were performed as described by De Vleesschauwer et al. (2012) with some modifications. Briefly, total protein samples were extracted from 3-week-old rice leaf samples with 2x extraction buffer (1 mM EDTA, 10% glycerol, 1% Triton X-100, 7 mM β-mercaptoethanol, 100 mM NaF, 1 mM $NaVO_3$, 1 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, 10 mM N-ethylmaleimide, protease inhibitor). Following quantification using a Bradford assay (Bio-Rad), protein extracts were mixed with an equal volume of 2x sample buffer (150 mM Tris-HCl, pH 6.8, 2% w/v SDS, 10% w/v glycerol, 0.01% w/v bromophenol, 0.1 M w/v dithiothretol), boiled for 5 min, separated on 10% SDS-PAGE gels, and transferred onto a nitrocellulose membrane. Polyclonal anti-SnRK1 antibody and goat anti-rabbit HRP-conjugated secondary antibodies were used to quantify SnRK1 accumulation. Peroxidase activity was detected by electrochemiluminescence (ECL) using

Super-Signal West Pico Chemiluminescent substrate (Thermo Scientific) according to the manufacturer's instructions.

Hormone quantification

Six-week-old T65 seedlings were inoculated with PXO99 as described above. Approximately 3-cm-long leaf segments right next to the inoculation site were harvested from mock-infected and *Xoo*-inoculated plants at 1, 2, 4 and 8 dpi. Three biological replicates were sampled, each replicate representing a pooled sample from at least four individual plants. CK measurements were carried out using ultra-performance liquid chromatography (UPLC) tandem mass spectrometry (AQITY UPLC System/Quattro Ultima Pt; Waters, Milford, MA, USA) with an ODS column (AQITY UPLC BEH C18, 1.7 µm, 2.1 by 100mm; Waters, Milford, MA, USA). Sample preparation and UPLC analysis were exactly as described in Kojima et al. (2009).

Table 5.2 Sequences of qRT-PCR primers used in this study

Locus ID	Gene name	Forward sequence	Reverse sequence
LOC_Os03g08020.1	*eElf1a*	TTTCACTCTTGGTGTGAAGCAGAT	GACTTCCTTCACGATTTCATCGTAA
LOC_Os04g57720.1	*OsRR6*	TCCTCAAGCGCGTCAAGGAATCG	TGATCCAACATGGCGAGCGAGC
LOC_Os02g35180.1	*OsRR2*	TGGCAGGACTAGCCATGGTGATG	TGCTGCCATTGGACCATCTGTGC
LOC_Os01g09800.1	*OsCKPrx*	CACGCCTAAGCCTCGGATTA	TCAGTGAGCAGCATCCTGACTAG
LOC_Os05g25770.1	*OsWRKY45*	GGACGCAGCAATCGTCCGGG	CGGAAGTAGGCCTTTGGGTGC
LOC_Os09g25070	*OsWRKY62*	AGCTTACTTCCGCTGCGCATTC	GCGACGAATTCGGTTGTCTGCG
LOC_Os02g56120.1	*OsIAA9*	CAACGACCACCAAGGCGAGAAG	CCAGGCAACCAAAACCGAGCTG

Chapter 6

Phytohormone-mediated interkingdom signaling shapes the outcome of rice-*Xanthomonas oryzae* pv. *oryzae* interactions

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Abstract

Plant hormones are well known to play key roles in the immune signaling network that is activated upon pathogen perception. In contrast, little is known about whether hormones also impinge on the virulence machinery of plant pathogens, similar to what has been reported in animal systems. Here, we tested the hypothesis that hormones fulfill dual roles in plant-microbe interactions by orchestrating host immune responses, on the one hand, and modulating microbial virulence traits, on the other. Employing the rice-*Xanthomonas oryzae* pv. *oryzae* (*Xoo*) interaction as a model system, we show that *Xoo* uses the classic immune hormone salicylic acid (SA) as a quorum sensing (QS) agonist. Despite repressing swimming motility, NaSA induced production of the Diffusible Signal Factor (DSF) and Diffusible Factor (DF) QS signals, with resultant accumulation of xanthomonadin and extracellular polysaccharide. In contrast, abscisic acid (ABA), which favors infection by *Xoo*, had little impact on DF- and DSF-mediated QS, but promoted bacterial swimming via the LuxR solo protein OryR. Cytokinin (CK), however, showed little effect on any virulence trait that we checked. Moreover, we found both DF and DSF to influence SA- and ABA-responsive gene expression *in planta*. Together our findings indicate that the rice SA and ABA signaling pathways cross-communicate with the *Xoo* DF and DSF QS systems and underscore the importance of bidirectional interkingdom signaling in molding plant-microbe interactions.

Introduction

Bacterial leaf blight (BLB) is one of the most devastating diseases on rice, causing annual yield losses up to 60% (Niño-Liu et al. 2006). The causal agent, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a rod-shaped, obligately aerobic, gram-negative bacterium that is motile by means of a single polar flagellum. Like many other *Xanthomonas* species, *Xoo* produces a wide variety of virulence factors to protect itself and inflict disease. These factors include extracellular polysaccharides (EPS), lipopolysaccharides, adhesins, cell wall degrading enzymes, and type III effectors (Shen and Ronald 2002; Büttner and Bonas 2010). Most of these traits are under tight control of quorum sensing (QS) regulatory systems.

QS is a cell-to-cell communication system by which bacteria track changes in cell density and adjust gene expression accordingly. Central to QS is the production, detection, and response to extracellular signal molecules called autoinducers (AIs). At low cell density, bacteria produce basal levels of AIs, which subsequently diffuse away in the environment, preventing detection by bacterial receptor proteins. However, as the population density

reaches a certain threshold level, accumulated AIs are easily detected, setting off a variety of biological processes including EPS biosynthesis, motility, competence, and virulence factor secretion (von Bodman et al. 2003; Atkinson and Williams 2009; von Bodman et al. 2008). As such, QS enables unicellular bacteria to act as multicellular organisms and invest in energy-consuming processes only when the impact of these processes on the environment or on a host will be maximized.

It is well-established that *Xoo* produces different types of AIs, including the Diffusible Signal Factor (DSF) molecule *cis*-11-methyl-2-dodecenoic acid (Barber et al. 1997; Deng et al. 2005; Jeong et al. 2008; Wang et al. 2004; Zhao et al. 2011). Originally identified in *Xanthomonas campestris* pv. *campestris* (*Xcc*), DSF and its derivatives represent a relatively novel family of QS signals that is widely conserved among Gram-negative bacterial species. So far, 13 structurally different DSF-family signals have been identified of which 6 are functionally characterized (Deng et al. 2005). Biosynthesis of DSF depends on *rpfB* and *rpfF*, which encode a putative long chain fatty acyl CoA ligase and a putative enoyl-coA hydratase, respectively (Barber et al. 1997; Zhao et al. 2011). Downstream of DSF synthesis, perception and transduction of DSF signals occurs through a conserved phosphorelay mechanism governed by the RpfC/RpfG two-component system (Ryan et al. 2006; He et al. 2006; Slater et al. 2000). Interestingly, RpfC not only senses DSF, but also negatively regulates DSF biosynthesis via direct protein-protein interactions (Cheng et al. 2010), while RpfG transmits DSF signals by influencing the level of the secondary QS messenger cyclic-di-GMP. Consistent with DSF signaling playing a vital role in *Xoo* virulence, null mutants of *rpf*C and *rpf*G display reduced xylanase activity, swimming ability and EPS synthesis, and consequently, cause little disease when inoculated on rice plants (Jeong et al. 2008).

In addition to DSF-governed QS, there is evidence for a second QS circuit in *Xoo* that is mediated by a Diffusible Factor (DF) characterized as 3-hydrobenzoic acid (3-HBA). As in *Xcc*, this DF QS system has been found to regulate the production of yellow, membrane-bound pigments, known as xanthomonadins (Zhou et al. 2013b). Besides being a diagnostic characteristic of the genus (Kennedy and En 1977; Jenkins and Starr 1982), xanthomonadins play essential roles in protecting bacteria from photobiological and peroxidative damage and are involved in epiphytic survival and systemic plant infection (Poplawsky et al. 2000; Kennedy and En 1977; Rajagopal et al. 1997). Goel et al. (2002) demonstrated that xanthomonadin synthesis in *Xoo* strain BXO1 is encoded by a 21 kb gene cluster that contains seven transcriptional units, designated *pigA* to *pigG* (Goel et al. 2002). Analysis of the *pigB* DNA sequence revealed the presence of two open reading frames, the second one (*xanB2*) being responsible for synthesis of DF (Poplawsky and Chun 1997; He et

al. 2011). Recent results indicate that XanB2 also hydrolyzes chorismate to produce 4-HBA, which is mainly used as a precursor for coenzyme Q (CoQ) biosynthesis (Zhou et al. 2013b).

Signaling pathways in plant are typically controlled by small-molecule hormones. Upon pathogen attack, plants synthesize a complex blend of hormones. This so-called hormone signature is well known to play a key role in the orchestration of plant immune responses and to determine the specific nature of the defense mechanism triggered (De Vos et al. 2005; Pieterse et al. 2012). However, exciting new developments suggest that plant hormones not only steer plant immune responses, but also may influence the pathogen's virulence machinery. For instance, a recent study showed that SA reduces virulence of *Agrobacterium tumefaciens* by inhibiting the VirA/G two-component system (Yuan et al. 2007b). Moreover, in the opportunistic human pathogen *Pseudomonas aeruginosa*, SA was reported to reduce the production of several virulence factors including motility, biofilm formation and AI production (Prithiviraj et al. 2005; Chow et al. 2011; Bandara et al. 2006).

In view of these findings, we hypothesized that plant hormones may fulfill a dual role in plant-bacteria interactions, modulating plant immune responses, on the one hand, and acting as (ant)agonists of bacterial QS systems on the other hand. To test this hypothesis, we investigated the effect of the plant hormones abscisic acid (ABA), cytokinin (CK) and salicylic acid (SA) on several QS-regulated virulence traits of *Xoo*, including motility, production of EPS and xanthomonadin synthesis. SA is a positive regulator of resistance to *Xoo* (De Vleesschauwer et al., 2013), whereas ABA antagonizes SA and acts as a susceptibility-enhancing hormone that is usurped by *Xoo* in order to cause disease (Xu et al. 2013). CK, however, showed little effect on the virulence factors that we checked. Reversely, we also tested the effect of the *Xoo* QS signals DSF and DF on the rice SA and ABA pathways. Together, our findings favor a scenario whereby SA and ABA cross-communicate with the DFS and DF QS circuits to modulate *Xoo* virulence and underscore the importance of bidirectional interkingdom signaling in molding plant-bacteria interactions.

Results

NaSA suppresses bacterial swimming in a dose-dependent manner

Mounting evidence indicates that SA is an important player in the activation of rice defenses against *Xoo* (Yuan et al. 2007a; De Vleesschauwer et al. 2013). In a first attempt to test whether SA also exerts direct effects on the QS machinery of *Xoo*, we evaluated the effect of SA on the swimming ability of *Xoo* strain XKK12 (pPIP122). Unless specified otherwise, this

strain was routinely used throughout this paper. Since SA showed strong inhibition of bacterial growth in both liquid and solid medium in our preliminary trials, all following experiments were performed using sodium salicylate (NaSA), which had no significant impact on XKK12 growth rates on (semi-)solid agar plates (data not shown). Swim plates indicated that XKK12 is motile in swimming medium, reaching a diameter of about 20 mm after 4-6 days of incubation at 25 °C. As shown in Figures 6.1A to 6.1C, NaSA at concentrations of 20 µM, 0.1 mM and 0.5 mM had little or no significant effect on the swimming ability of XKK12, whereas 1 mM and 2 mM of NaSA significantly reduced swimming. SA concentrations in *Xoo*-inoculated rice leaves range from 5 to 30 µg/g fresh weight, which corresponds to approximately 0.5 to 3.0 mM SA (Liu et al. 2012; Huang et al. 2006). Supplying bacteria with 1 or 2 mM NaSA thus resembles the amount of SA encountered by *Xoo in planta*, demonstrating the physiological relevance of these concentrations.

Motility of *Xoo* is under control of the DSF QS system, with DSF signaling either promoting or suppressing swimming depending on the bacterial strain (Rai et al. 2012; Jeong et al. 2008; He et al. 2010). Under our experimental conditions, 3 µM DSF, which is the lowest concentration reported to be effective in *Xoo* (He et al. 2010), significantly increased swimming of XKK12 (Figure 6.1D). Interestingly, co-application of 3 µM DSF and 1 mM NaSA alleviated the swimming-promoting effect of single DSF treatments, while 2 mM NaSA completely abolished it (Figure 6.1D). Together these results suggest that NaSA suppresses swimming of XKK12 in a concentration-dependent manner, possibly by antagonizing DSF-mediated signaling.

Swimming motility of *Xoo* was previously shown to be controlled by OryR, which is a sub-family of the *N*-acyl homoserine lactone (AHL) responding LuxR regulators (Ferluga and Venturi 2009; Gonzalez et al. 2013). Since there is not a cognate LuxI-family AHL synthase in the *Xoo* genome, and OryR has been designated as a LuxR 'solo' protein which does not bind AHLs but responds to a yet unknown plant low-molecular-weight compound(s). To assess whether NaSA suppresses swimming in an OryR-dependent manner, we tested the effect of NaSA on WT bacteria and the *oryR* knockout mutant (*oryR*$^-$). Consistent with earlier findings by Gonzalez et al. (2013) demonstrating that the effect of OryR on bacterial swimming is only observed upon addition of macerated rice extract to the medium, WT and *oryR*$^-$ mutant bacteria swam to a similar extent when grown on control pates. Perhaps more surprisingly, the *oryR*$^-$ mutant proved to be non-responsive to DSF-induced swimming, while NaSA treatment turned out to be equally effective in the WT and mutant background (Figure

6.1D). Thus, OryR seems to be indispensable for DSF-induced swimming, whereas NaSA may suppress swimming independently of OryR.

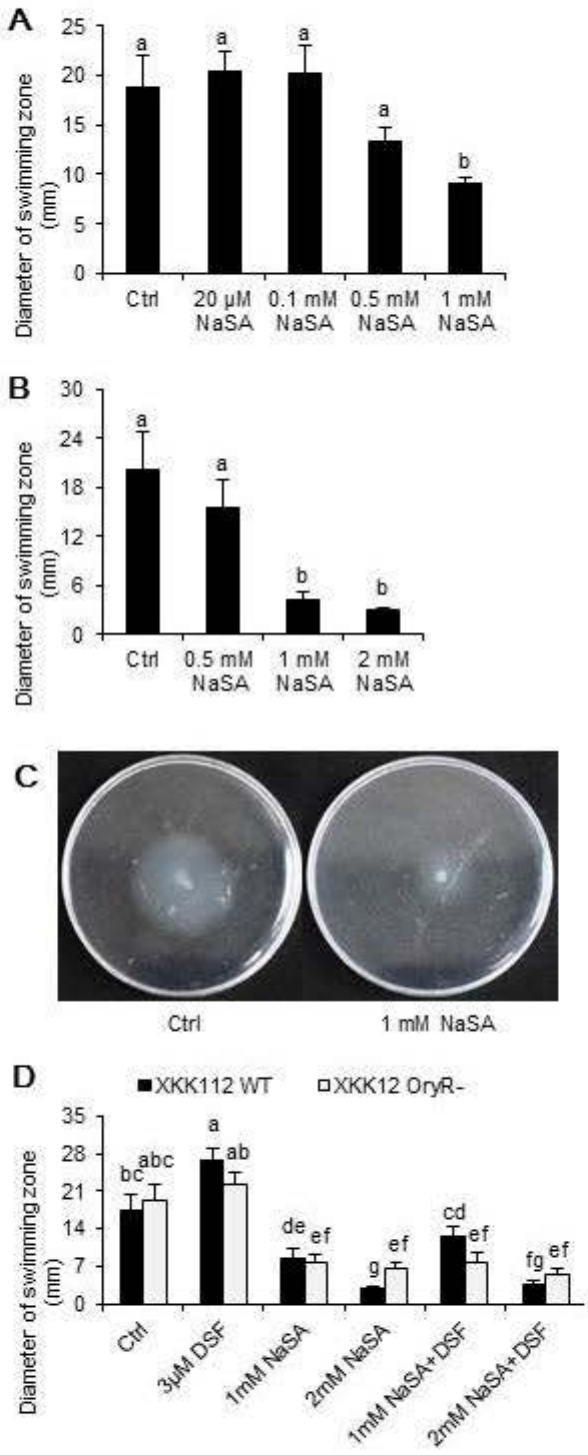

Figure 6.1 NaSA suppresses basal and DSF-induced swimming motility of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) strain XKK12 (pPIP122). (A) and **(B)** Various concentrations of NaSA were added to swimming plates prior to inoculation with 3 µl XKK12 WT (pPIP122) suspension (10^9 CFU/ml). The plates were incubated for four days at 25 °C and evaluated by measuring the diameter of the swimming zone. Data are means ± SE. Different letters indicate statistically significant

differences (Mann-Whitney: n ≥ 9; α = 0.05). **(C)**, Phenotype of XKK12 WT (pPIP122) on swimming plates containing 0 (left) or 1 mM NaSA (right). **(D),** Effects of 1 mM NaSA, 2 mM NaSA and/or 3 µM DSF on the swimming of XKK12 WT (pPIP122) and *oryR* knockout mutant (pPIP122) (*oryR*$^-$) bacteria. Data are means ± SE. Different letters indicate statistically significant differences (Mann-Whitney: n ≥ 9; α = 0.05).

NaSA induces EPS production in XKK12 via OryR

In many bacteria, extracellular polysaccharide (EPS) is a key determinant of biofilm formation and, hence, an inhibitory factor of motility (Dow et al. 2003; Rosenberg et al. 2013). When XKK12 was grown on solid PSA plates, NaSA showed no effect on bacterial growth but significantly enhanced EPS synthesis (Figure 6.2A). In several preliminary assays liquid XKK12 cultures grown in the presence of 1 mM NaSA also consistently produced more EPS than the control treatment at 24 h post inoculation (OD control 3.15±0.53, OD NaSA treatment 2.78±0.53, EPS production control 0.40±0.07 mg/OD, EPS production NaSA treatment 0.75±0.09 mg/OD – data from 5 different experiments). Therefore, and given the tractability of liquid assays, all follow-up experiments were performed using liquid XKK12 cultures.

Previous work revealed that EPS production is impaired in the *Xoo rpf*F knockout mutant but restored by exogenous addition of DSF (He et al. 2010; Rai et al. 2012; Jeong et al. 2008; Chatterjee and Sonti 2002), suggesting a positive role for DSF in regulating EPS production in *Xoo*. Consistent with these results and as shown in Figure 6.2B, 3 µM DSF significantly increased EPS synthesis in XKK12. A similar promoting effect was observed for 1 mM NaSA, whereas co-application of both compounds had no additive effect. Interestingly, NaSA-induced EPS synthesis was much less pronounced in the *oryR*$^-$ mutant background but restored in the *oryR*$^-$ complemented strain (Figure 6.2C), suggesting that NaSA stimulates EPS production in XKK12 in an OryR-dependent manner. Consistent with this hypothesis, qRT-PCR analysis showed that 1 mM NaSA strongly up-regulated the expression of both *oryR* and the EPS biosynthesis gene *gumG* compared with control treatments (Figures 6.2D and 6.2E). Moreover, β-glucuronidase promoter assays revealed that NaSA also causes an approximate 50% increase in *oryR* promoter activity (Figure 6.2F).

NaSA stimulates DSF signaling pathway by inducing DSF biosynthesis

The finding that NaSA induces transcription of the LuxR solo *oryR* led us to analyze whether NaSA likewise influences the DSF QS system. For this purpose, we first tested the effect of NaSA on the expression of various genes located in the DSF signaling cascade. *rpfF*, *rpfC* and *rpfG* are DSF biosynthesis, response and signaling genes, respectively (Jeong et al. 2008; Guo et al. 2013), while *pilA* encodes an adhesion protein involved in biofilm formation

and motility (Jeong et al. 2008). Remarkably, all of these genes were several fold up-regulated by 1 mM NaSA treatment (Figure 6.3A), suggesting that NaSA activates the entire DSF pathway, possibly by inducing DSF biosynthesis. To test this hypothesis, we quantified the effect of NaSA on steady-state production levels of DSF and the DSF-like signals BDSF and CDSF using HPLC (He et al. 2010). As shown in Figure 6.3B, NaSA did not significantly influence BDSF production but caused a nearly 50% increase in DSF levels, while CDSF remained below the detection limit.

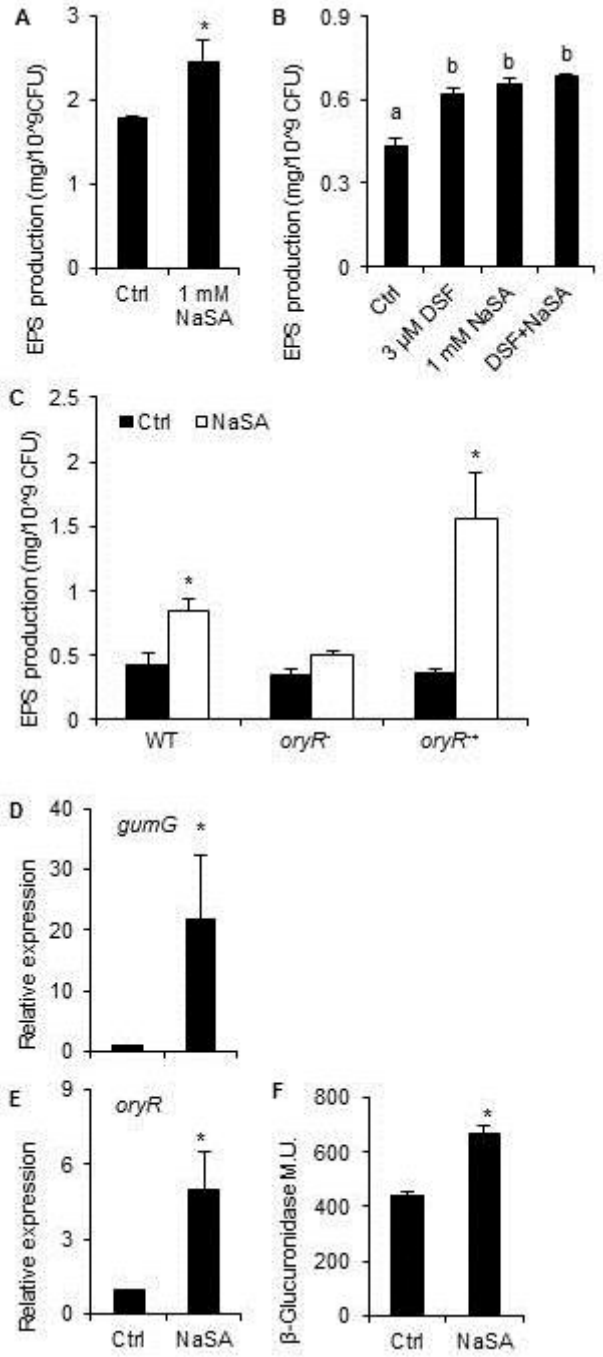

Figure 6.2 NaSA stimulates EPS synthesis in XKK12 WT (pPIP122) in an OryR-dependent manner. (A), EPS production of XKK12 WT (pPIP122) grown on PSA plates with or without 1 mM

NaSA. Data are means ± SE of three plates. Asterisks indicate statistically significant differences compared to the control (T-test: n = 3; α = 0.05). **(B)**, Effects of 1 mM NaSA and/or 3 µM DSF on EPS production of XKK12 WT (pPIP122) grown in PY broth. Data are means ± SE of three replicates. Asterisks indicate statistically significant differences compared to the control (Tukey: n = 3; α = 0.05). **(C)**, Effect of 1 mM NaSA on EPS production of XKK12 WT, *oryR*$^{-}$ and *oryR*$^{-}$ complemented strain (*oryR*$^{-+}$) grown in PY broth. Data are means ± SE of three independent experiments. Different letters indicate significant differences (T-test: n = 3; α = 0.05). **(D)** and **(E),** Effect of 1 mM NaSA on expression profiles of *oryR* and the EPS biosynthesis gene *gumG* in PY-grown XKK12 WT (pPIP122). Data are means ± SE of two technical and two biological replicates. Asterisks indicate statistically significant differences compared to the control (T-test: n = 4; α = 0.05). **(F)**, *oryR* gene promoter activity in XKK12 WT harboring the reporter plasmid pORYR122 (Ferluga and Venturi, 2009) grown in PY broth supplemented or not with 1 mM NaSA. Data are means ± SE of three replicates from a representative experiment. The experiment was repeated twice with similar results. Different letters indicate significant differences (T-test: n = 3; α = 0.05).

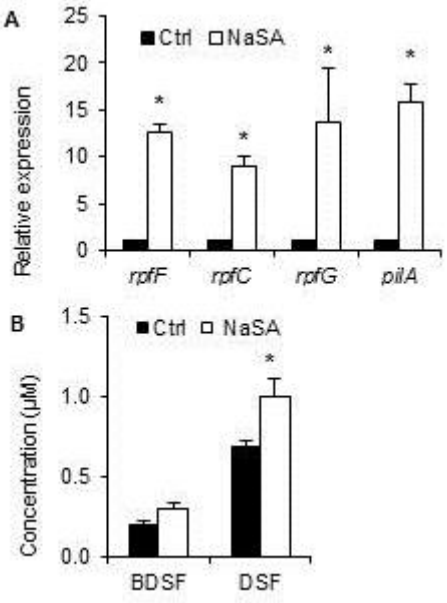

Figure 6.3 Effect of NaSA on DSF biosynthesis and signaling. (A), Expression of DSF biosynthesis and responsive genes *rpfF*, *rpfC*, *rpfG*, and adhesin-encoding *pilA* in XKK12 WT (pPIP122) grown in PY broth supplemented or not with 1 mM NaSA. Data are means ± SE of two technical and two biological replicates. Asterisks indicate statistically significant differences compared to the control (T-test: n = 4; α = 0.05). (B), Quantification of DSF and BDSF produced by XKK12 WT (pPIP122) grown in PY broth containing 0 or 1 mM NaSA. Data are means ± SE of three independent experiments. Asterisks indicate statistically significant differences compared to the control (T-test: n = 3; α = 0.05).

NaSA induces 3-HBA and 4-HBA production in XKK12

In addition to OryR and the DSF QS system, *Xoo* is equipped with an alternative QS circuit that is mediated by the DF signal and controls production of xanthomonadin. Interestingly,

XKK12 bacteria grown on PSA plates containing 1 mM NaSA produced about 60% more xanthomonadin compared with the control (Figure 6.4A and 6.4B). Consistent with these findings, NaSA-supplemented bacteria also displayed increased expression of the xanthomonadin and DF biosynthesis gene *xanB2* (Figure 6.4C). Moreover, HLPC-based measurements revealed that NaSA stimulates the production of 3-HBA (DF) and 4-HBA in a dose-dependent manner (Figure 6.4D). Hence, besides inducing DSF signaling, NaSA also appears to activate the DF QS circuit, with resultant accumulation of xanthomonadin.

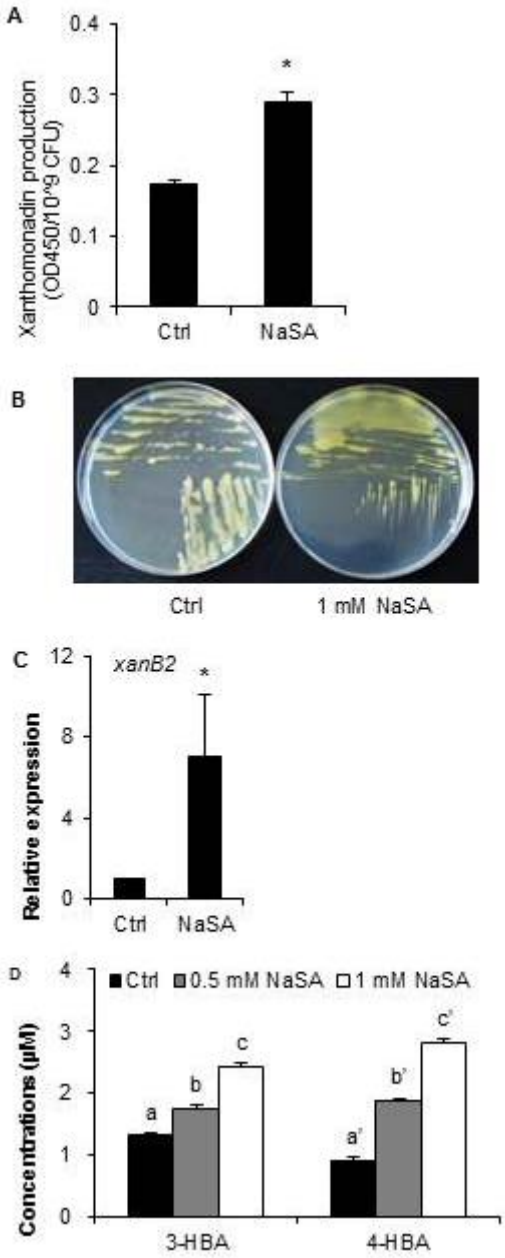

Figure 6.4 NaSA induces the DF QS circuit of *Xoo* with resultant accumulation of xanthomonadin. (A), Effect of 1 mM NaSA on xanthomonadin production of XKK12 WT (pPIP122) grown in PY broth. Data are means ± SE of three plates. Asterisks indicate statistically significant differences compared to the control (T-test: n = 3; α = 0.05). (B), Yellow pigmentation of XKK12 WT (pPIP122) grown on PSA plates containing 0 (left) or 1 mM NaSA (right). (C), Expression of

xanthomonadin and DF biosynthesis gene *xanB2* in XKK12 WT (pPIP122) grown in PY broth containing 0 or 1 mM NaSA. Data are means ± SE of two technical and two biological experiments. Asterisks indicate statistically significant differences compared to the control (T-test: n = 4; α = 0.05). (D), Quantification of 3-HBA (DF) and 4-HBA produced by XKK12 WT (pPIP122) grown in PY broth supplemented or not with 0.5 or 1 mM NaSA. Data are means ± SE of two technical and two biological replicates. Different letters indicate significant differences (Tukey: n = 4; α = 0.05).

ABA promotes swimming of XKK12 in an OryR-dependent manner

Previously, we showed that ABA antagonizes SA signaling in rice, thus favoring BLB disease development (Xu et al. 2013). In a first attempt to investigate whether ABA, like SA, acts as a QS (ant)agonist, we tested the effect of increasing concentrations of ABA on the swimming ability of XKK12. After 4 days of incubation, bacteria cultured on plates containing either 10 µM, 50 µM or 100 µM of ABA showed enhanced swimming compared to the solvent-treated controls, whereas 1 µM ABA had no significant effect (Figure 6.5A and 6.5B). Since none of these concentrations had a significant impact on the growth characteristics of XKK12 (data not shown), these data show that ABA concentrations above 1 µM promote swimming without any clear dose-response.

Given the positive role of OryR in promoting bacterial swimming, we next compared the effect of 50 µM ABA on WT and *oryR*$^-$ bacteria. Interestingly, ABA treatment was ineffective in the *oryR*$^-$ background whereas it significantly enlarged the swimming zone in WT bacteria. Consistent with previous findings, complementing the *oryR*$^-$ mutant by introducing the *oryR* gene in the high-copy plasmid pBBROryR significantly decreased swimming as compared to WT bacteria, a phenomenon which is likely explained by the overexpression of *oryR* in a multicopy plasmid and the self-negative regulation of *oryR* (Gonzalez et al. 2013). Nevertheless, complementation of *oryR*- did restore ABA-induced swimming, indicating that OryR is indispensable for ABA-mediated swimming (Figure 6.5C).

To test whether ABA, like SA, affects *oryR* expression, we next investigated the transcriptional behavior of *oryR* in XKK12 WT pPIP122 grown in PY broth containing 50 µM ABA. As shown in Figure 6.5D, ABA had little effect on the expression of *oryR* and, according to our β-glucuronidase assays, also failed to induce *oryR* promoter activity (Figure 6.5E). Moreover, growing bacteria in the presence of ABA did not significantly alter the expression of the OryR-regulated flagellar genes *flgL*, *flgF*, *flhF* and *cheZ* (Gonzalez et al. 2013), suggesting that ABA-induced swimming of *Xoo* does not involve transcriptional activation of *oryR* or its target genes (Figure 6.5F).

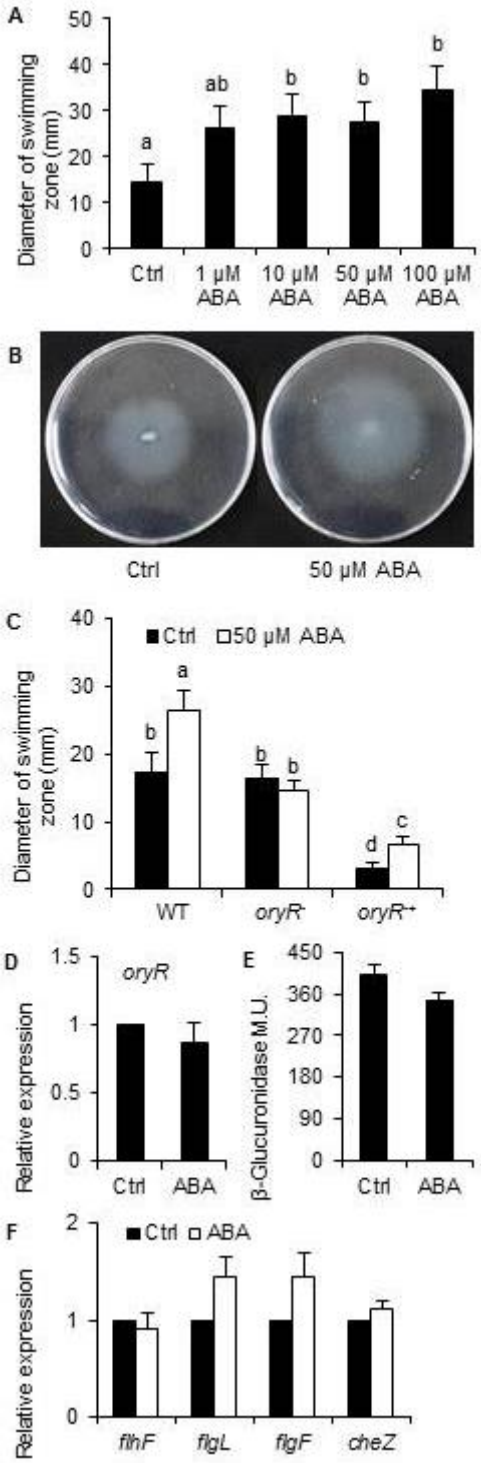

Figure 6.5 Abscisic acid (ABA) promotes swimming of XKK12 WT (pPIP122) via the LuxR solo OryR. (A), Effect of different ABA concentrations on swimming motility of XKK12 WT (pPIP122). Equivalent volumes of the ABA solvent ethanol were added to control (Ctrl) treatments. Data are means ± SE. Different letters indicate statistically significant differences (LSD: $n \geq 9$; $\alpha = 0.05$). (B), Phenotype of XKK12 WT (pPIP122) on swimming plates containing 0 (left) or 50 µM ABA (right). (C), Effect of 50 µM ABA on the swimming of XKK12 WT, *oryR*$^{-}$ and the complemented stain *oryR*$^{-+}$. Data are means ± SE of three independent experiments. Different letters indicate statistically significant differences (Mann-Whitney: $n \geq 18$; $\alpha = 0.05$). (D)**,** Expression of *oryR* in XKK12 WT (pPIP122) grown in PY broth with or without 50 µM ABA. Data are means ± SE of two technical replicates and two biological replicates. There were no significant differences between control (Ctrl) and ABA treatments.

(T-test: n = 4; α = 0.05). (E), *oryR* gene promoter activity in XKK12 WT harboring the pORYR122 reporter plasmid and grown in PY broth supplemented or not with 50 µM ABA. Data are means ± SE of three replicates from a representative experiment. There were no significant differences between control (Ctrl) and ABA treatments (T-test: n = 3; α = 0.05). Repetition of experiments led to results very similar to those shown. (F), Expression of OryR-regulated flagellar genes in XKK12 WT (pPIP122) grown in PY broth in response to 50 µM ABA. Data are means ± SE of two technical and two biological replicates. There were no significant differences between control (Ctrl) and ABA treatments. (T-test: n = 4; α = 0.05).

ABA has little impact on the DSF and DF QS circuits

The finding that NaSA induces several genes located in the DSF and DF QS pathways (Figures. 6.2, 6.3 and 6.4) prompted us to check whether ABA exerted a similar effect. However, as shown in Figure 6.6A, ABA had no statistically significant impact on the expression of any of the DF and DSF-related genes tested. ABA also failed to significantly alter the synthesis of BDSF and DSF, but slightly repressed 3-HBA and weakly enhanced 4-HBA synthesis, which has also been implicated in EPS and xanthomonadin regulation (Zhou et al. 2013a) (Figures 6.6B and 6.6C). Nevertheless, we found ABA to be ineffective in both EPS and xanthomonadin assays (data not shown). Except for stimulating swimming via a yet to be defined mechanism, ABA therefore seems to have little effect on the DSF and DF QS circuits, neither on the virulence traits mediated by them.

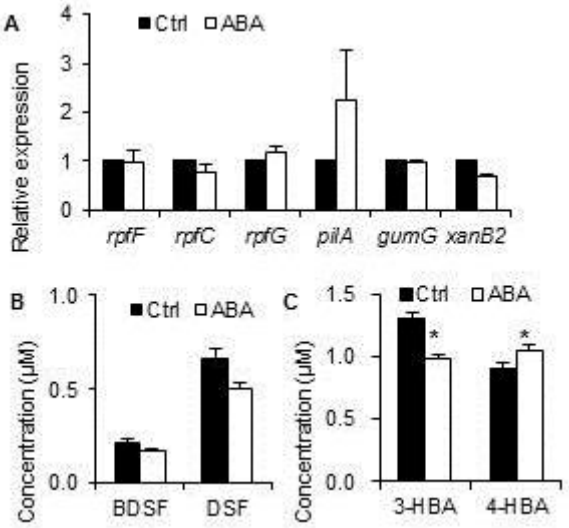

Figure 6.6 ABA has little impact on the DSF- and DF-type QS circuits in *Xoo*. (A), Expression of DSF biosynthesis and responsive genes *rpfF*, *rpfC*, and *rpfG*, EPS biosynthesis gene *gumG*, adhesion gene *pilA*, and xanthomonadin biosynthesis gene *xanB2*, in XKK12 WT (pPIP122) grown in PY broth in response to 50 µM ABA. Data are means ± SE of two technical and two biological replicates. There were no significant differences between control (Ctrl) and ABA treatments. (T-test: n = 4; α = 0.05). (B) and (C), Quantification of DSF and DF produced by XKK12 WT (pPIP122) grown in PY broth with or without 50 µM ABA. Data are means ± SE of three independent experiments. Asterisks indicate statistically significant differences compared to the control (T-test: n = 4; α = 0.05).

CK has little impact on QS-regulated virulence factors of *Xoo*

As discussed in Chapter 5, CK promotes BLB development on rice. Hence, we sought to examine whether CK, like ABA and SA, function as a QS stimuli for *Xoo*. To start, we tested the effect of 50 μM kinetin on swimming ability of XKK12. As shown in Table 6.1, 50 μM kinetin had little influence on swimming motility of XKK12. Moreover, we applied 50 μM kinetin in PY broth to grow XKK12 and quantified the production of EPS and xanthomonadin. However, these two virulence traits were not affected by kinetin neither (Table 6.1). In line with these results from bioassays, expression profiles of the corresponding genes were not altered by the kinetin treatment (data not shown). Together, CK is unlikely to affect these QS-regulated virulence traits of *Xoo*.

Table 6.1 Effect of 50 μM kinetin on swimming motility, EPS and xanthomonadin production of XKK12 (pPIP122). Data presented are means ± SE.

	Ctrl	50 μM kinetin
Diameter of swimming zone (mm) (n⩾9)	24.750 ± 0.870	21.615 ± 2.747
EPS production (mg/10^9 CFU) (n=3)	0.411 ± 0.006	0.359 ± 0.021
Xanthomonadin production (OD445/10^9 CFU) (n=4)	0.157 ± 0.012	0.156 ± 0.001

DSF and DF differentially impact rice ABA and SA signaling pathways

Apart from coordinating expression of microbial virulence genes, bacteria-produced QS signals have also been shown to interfere with the host hormone signaling circuitry (Schikora et al. 2011; Mathesius et al. 2003). Therefore and given the importance of SA-ABA signal interactions in BLB resistance (Xu et al. 2013), we evaluated the impact of exogenously administered DSF and DF on rice SA and ABA signaling pathways. To this end, detached leaves of 6-week-old Taipei plants were treated with either low (1 μM) or high (50 μM) concentrations of DSF and DF and tested for expression of several ABA and SA-responsive marker genes. As shown in Figure 6.7, expression of the SA marker genes, *OsWRKY45*, *OsWRKY62* and *OsNPR1* was only weakly responsive to either 1 or 50 μM DSF, suggesting that DSF has little, if any, impact on the rice SA pathway. In addition, 50 μM DSF barely altered the expression of the ABA marker gene *OsRab16* at either 8 or 24 hours post treatment while it had a significant albeit minor suppressive effect on transcription of another marker gene *OsLip9* (hpt; Figures 6.7D and 6.7E). Suppression of *OsLip9* was also observed in response to DF at 8 hpt, whereas *OsRab16* was upregulated by 5 μM DF at 24 hpt (Figures. 6.7I and 6.7J). In addition, both 5 μM and 50 μM DF treatments significantly

lowered transcription of *OsNPR1*, *OsWRKY45* and *OsWRKY62* at 8 but not at 24 hpt, suggesting that DF is able to transiently suppress SA signaling.

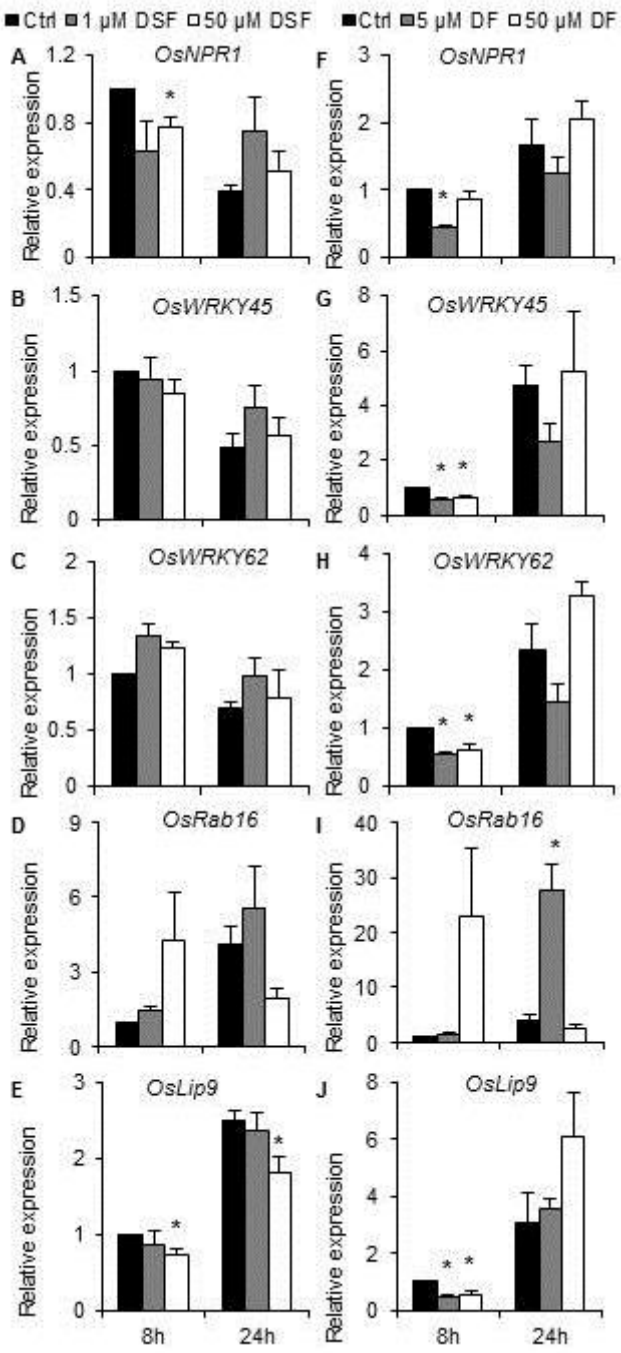

Figure 6.7 Effects of DSF and DF on ABA and SA signaling pathways in rice. 6-week-old Taipei leaf segments were incubated in aqueous solutions containing 1 or 50 µM DSF (A to E) or DF (F to J), or equivalent volumes of solvent (Ctrl), and sampled at 8 and 24 hours post treatment (hpt). Expression of the ABA responsive genes *OsLip9* and *OsRab16* and the SA marker genes *OsWRKY45*, *OsNPR1* and *OsWRKY62*, was determined by qRT-PCR. Data are means ± SE of two technical and two biological replicates. Asterisks indicate statistically significant differences compared to the control (T-test: n = 4; α = 0.05). Repetition of experiments led to results very similar to those shown.

Discussion

The interaction between pathogens and their host plants has been the subject of intense research. Contrary to the now large body of evidence demonstrating the pivotal role of hormones in orchestrating the plant immune signaling network, surprisingly little is known about whether hormones also impinge on the virulence machinery of plant pathogens, similar to what has been reported in animal systems. In one of the most notable examples, the enterohemorrhagic *Escherichia coli* (EHEC) serotype O157:H7, a pathogen responsible for outbreaks of bloody diarrhea in several countries, was shown to exploit the mammalian hormone epinephrine in order to activate its QS machinery (Sperandio et al. 2003). Similarly, several reports in the literature have revealed a negative impact of the plant hormone SA on virulence gene expression in the opportunistic human pathogen *Pseudomonas aeruginosa* (Prithiviraj et al. 2005; Chow et al. 2011). In turn, several AHL QS factors have been shown to exert immunomodulatory activities in animal hosts (Williams 2007). These studies suggest that prokaryotic-eukaryotic communication occurs through bacterial QS factors and host hormones. Here we provide the first report of a similar phenomenon with respect to the plant pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), one of the most important threats of cultivated rice worldwide. Our findings favor a scenario wherein SA and ABA not only steer rice immune responses, but also cross-communicate with bacterial QS systems to regulate virulence traits in *Xoo*. Moreover, our results also imply reciprocal crosstalk with *Xoo* QS signals modulating rice SA and ABA signaling pathways. We propose that the dynamic nature and balance of such bidirectional interkingdom signaling is an important regulatory factor determining the outcome of plant-bacteria interactions.

SA activates the *Xoo* DSF and DF QS systems to coordinately regulate swimming, EPS and xanthomonadin synthesis

Swimming and EPS production are important virulence factors of *Xoo* and both traits are considered to be under control of QS. Several lines of evidence indicate that DSF signaling positively regulates EPS production, whereas the effect of DSF on swimming of *Xoo* appears to be strain-dependent (Jeong et al. 2008; Rai et al. 2012). Under our experimental conditions, exogenously administered DSF promoted both swimming and EPS production of strain XKK12, confirming the importance of DSF signaling in these processes (Figure 6.1D and 6.2A). NaSA, on the other hand, inhibited swimming but strongly promoted EPS synthesis and up-regulated various genes located both upstream (*rpf* gene cluster) and downstream (*pilA*) in the DSF signaling pathway (Figures 6.1, 6.2 and 6.3A). Intriguingly, we also found NaSA to strongly increase DSF production in liquid cultures (Figure 6.3B),

providing a mechanism for how NaSA may activate the entire DSF signaling circuit and increase EPS synthesis by stimulating DSF biosynthesis.

NaSA-induced DSF synthesis may seem contradictory in light of the positive role of DSF in swimming of XKK12 and the ability of NaSA to repress the latter process. However, regulation of flagellar motility in xanthomonads is a complex multi-step process involving over 40 genes that are regulated in a hierarchical manner (Lee et al. 2005; Aldridge and Hughes 2002). Kunin et al. (1995) previously demonstrated the ability of SA to block flagella biosynthesis in E. coli. Therefore, it is not inconceivable that NaSA transiently activates DSF signaling, but negatively affects swimming of XKK12 by an as yet unknown mechanism, which could involve repression of individual flagella-associated genes and/or post-transcriptional modifications.

In addition to setting off DSF QS signaling, our data also point towards a role of NaSA in activating the DF QS system. Genetic analysis revealed that DF (3-HBA), which is synthesized by the key metabolic enzyme XanB2, catalyzes the production of xanthomonadin pigments (Zhou et al. 2013a; He et al. 2011; Zhou et al. 2013b). Interestingly, xanthomonadin is synthesized from shikimate and chorismate, both of which are also important precursors for SA biosynthesis in plants (Silverman et al. 1995; Shah 2003). Although the structure of SA (2-HBA) is very similar to that of DF (3-HBA), SA failed to complement the *Xcc xanB2* knockout mutant (He et al. 2011). However, we found that NaSA enhances *xanB2* gene expression and, accordingly, triggers enhanced levels of DF and xanthomonadin (Figures 6.4B and 6.4C). Notably, DF is reported to be responsible for EPS production as well (Zhou et al. 2013a), suggesting that the induction of EPS by NaSA may derive at least in part from its positive effect on DF synthesis. NaSA also promoted the production of 4-HBA (Figure 6.4C), the second gene product of *xanB2* (Zhou et al. 2013b, 2013a). Unlike DF, 4-HBA is responsible for the biosynthesis of CoQ, which functions as a key cofactor in the aerobic respiratory electron transfer for energy generation and protects bacteria from peroxidative damage (Cluis et al. 2007). Given the importance of DF and 4-HBA in bacterial virulence (Zhou et al. 2013a, 2013b) and the well-described role of EPS and xanthomonadin in protecting bacteria from host-imposed stresses (Poplawsky et al. 2000; Rajagopal et al. 1997; Denny 1995), one interesting extrapolation is that SA not only serves as a plant resistance-inducing signal, but also ended up being exploited by *Xoo* as a QS agonist. Moreover, in view of the idea that bacteria possess intensity ‘switches’ that ensure timely expression of energy-intensive processes (Blocker et al. 2003), it is tempting to speculate that *Xoo* uses SA to ‘sense’ that is within the host vascular system and activate genes essential for plant colonization.

The LuxR-type solo OryR coordinates crosstalk between plant SA/ABA and *Xoo* QS circuits

Belonging to a sub-family of LuxR proteins that have the same modular structure of QS LuxRs but are devoid of a cognate LuxI AHL synthase, the so-called LuxR 'solo' OryR controls the expression of more than 300 bacterial genes and is important for the complete virulence of *Xoo* (Ferluga et al. 2007; Gonzalez et al. 2013). LuxR solos regulate target genes by either sensing endogenous QS factors or by 'eavesdropping' on exogenous factors produced by neighboring bacteria (Ahmer 2004). However, some solos can also respond to low-molecular weight compounds produced by plants (Patel et al. 2013). Biochemical studies have shown that OryR does not respond to a wide variety of AHL-type QS factors, but is solubilized in the presence of media supplemented with macerated rice leaves (Ferluga et al. 2007; Ferluga and Venturi 2009). This indicates that OryR most probably binds a plant-produced compound, the identity of which remains unclear. Intriguingly, our data suggest that OryR is involved in the perception and/or transduction of plant hormone signals with NaSA activating both gene expression and promoter activity of *oryR* (Figures 6.2E and 6.2F). Moreover, experiments with *oryR*$^{-}$ knockout strains revealed that OryR is indispensable for NaSA-induced EPS synthesis and ABA-promoted swimming. Although it does not follow that OryR necessarily acts as a bacterial 'hormone receptor' and, hence, directly binds NaSA and/or ABA, these findings do suggest that OryR plays a pivotal role in integrating, processing and transmitting plant and bacterial signal molecules, thus acting as a major hub for signal integration and pathway crosstalk. Nonetheless, it should be noted that OryR is not solely determinant for bacterial hormone responses as NaSA reduced swimming to similar extents in WT and *oryR*$^{-}$ mutant bacteria (Figure 6.1D). Moreover, unlike SA, ABA treatment had no significant impact on gene expression and promoter activity of *oryR* (Figures 6.5D and 6.5E), suggesting that ABA regulates swimming of XKK12 downstream of OryR. Gene expression experiments failed to show a substantial effect of ABA on any of the OryR-dependent flagellar genes we tested (Figure 6.5F). Since there are 17 motility-related genes that are positively regulated by OryR, it cannot be ruled out that ABA affects one or more genes that we did not check. Furthermore, ABA-mediated regulation of swimming may also occur at the post-transcriptional level. Deciphering the exact mechanism(s) by which ABA regulates OryR-dependent swimming is a key challenge for future research.

Xoo QS signals (DSF and DF) modulate SA and ABA signaling pathways in rice

In addition to plants producing AHL mimics that are able to act as agonists or antagonists to bacterial AHL QS systems, accumulating evidence indicates that bacterial QS factors modulate plant-microbe interactions by tapping into various plant signaling circuits (Bauer and Mathesius 2004; Hartmann et al. 2014). In one of the first examples, it was reported that treatment of *Medicago truncatula* with AHLs drives transcriptional reprogramming of extensive gene sets involved in host defense responses, primary metabolism, transcriptional regulation, protein processing, cytoskeletal activity, and plant hormone responses (Mathesius et al. 2003). Similarly, AHL-treated Arabidopsis displayed altered expression of selected hormone-responsive genes as well as significant changes in the plant's hormone balance, in particular an increased auxin/cytokinin ratio (von Rad et al. 2008). In common with these findings, our data revealed that low and high concentrations of DF but not DSF transiently suppress SA-responsive gene expression in detached rice leaves. Considering the similarity in chemical structure of DF (3-HBA) and SA (2-HBA), one may envision that *Xoo*-secreted DF not only facilitates bacterial cell-to-cell communication, but also disrupts host immune responses by acting as an SA mimic. With respect to the ABA pathway, DF showed ambivalent effects with both positive and negative outcomes being found depending on the gene tested (Figs. 7I and 7J). High concentrations of DSF, on the other hand, weakly but significantly suppressed ABA signaling. Although these results are consistent with a recent study showing that *Xcc* overcomes ABA-mediated stomatal immunity in Arabidopsis through a DSF-regulated virulence factor (Gudesblat et al. 2009), they may also seem conflicting with the previously reported role of ABA in promoting BLB disease development (Xu et al. 2013). However, given the tremendous increase in ABA-responsive gene expression in *Xoo*-infected rice leaves (up to 2,000-fold; Xu et al., 2013) and the rather weak effect of DSF on ABA signaling (less than 20% reduction relative to controls), one may question the biological significance of negative DSF-ABA interactions in determining the outcome of rice-*Xoo* interactions.

Bidirectional interkingdom signaling molds rice-*Xoo* interactions

Collectively, our data imply a potential cross-communication between the DF and DSF *Xoo* QS circuits and the rice SA and ABA signaling pathways. In Figure 6.8, we propose a spatiotemporal model illustrating the role and interplay of plant hormones and bacterial QS factors in regulating rice-*Xoo* interactions. We speculate that upon leaf entry, *Xoo* is faced with high levels of SA in the xylem, which restricts its growth and motility and is deployed by

rice as an efficient defense mechanism. *Xoo*, however, appears to perceive SA as a QS agonist with resultant activation of the DSF and DF QS systems, a process that at least partially depends on the LuxR solo protein OryR. SA-induced DF and DSF signaling in turn triggers production of EPS, xanthomonadin and CoQ, which collectively contribute to bacterial virulence. At this stage, *Xoo* are sessile and most likely growing as single colony encased within a biofilm. Via a yet-to-be-defined mechanism, successful *Xoo* bacteria then trick the plant into synthesizing ABA, which together with SA-induced DF contributes to suppression of SA signaling. As biofilms grow in size, cells that reside in the innermost layers of the biofilm may not have access to nutrients or may suffer from accumulation of toxic waste products. In either of these cases, or when environmental nutrients become limited, bacteria are likely to respond by returning to their planktonic mode of existence, thus inducing biofilm dispersal. With the aid of ABA and OryR and free from SA suppression, bacteria that are released from the biofilm swim to a further site, where they are again confronted with high SA levels. By repeating this process over and over again, *Xoo* bacteria may gradually spread along the rice xylem vessels, causing typical leaf blight symptoms as they progress. Although the specific mechanisms involved in various steps of the model remain to be elucidated, our data draw important inferences suggesting that *Xoo* responds to both a bacterial QS signaling system and a rice hormone signaling circuit to fine-tune virulence gene expression at different stages of infection. Moreover, besides reinforcing the earlier contention that QS is a bidirectional process influenced by both plant and microbial signals (Hartmann et al. 2014), our findings highlight the importance of plant hormones in modulating bacterial virulence and uncover interkingdom signaling as an important regulatory aspect of plant-bacteria interactions.

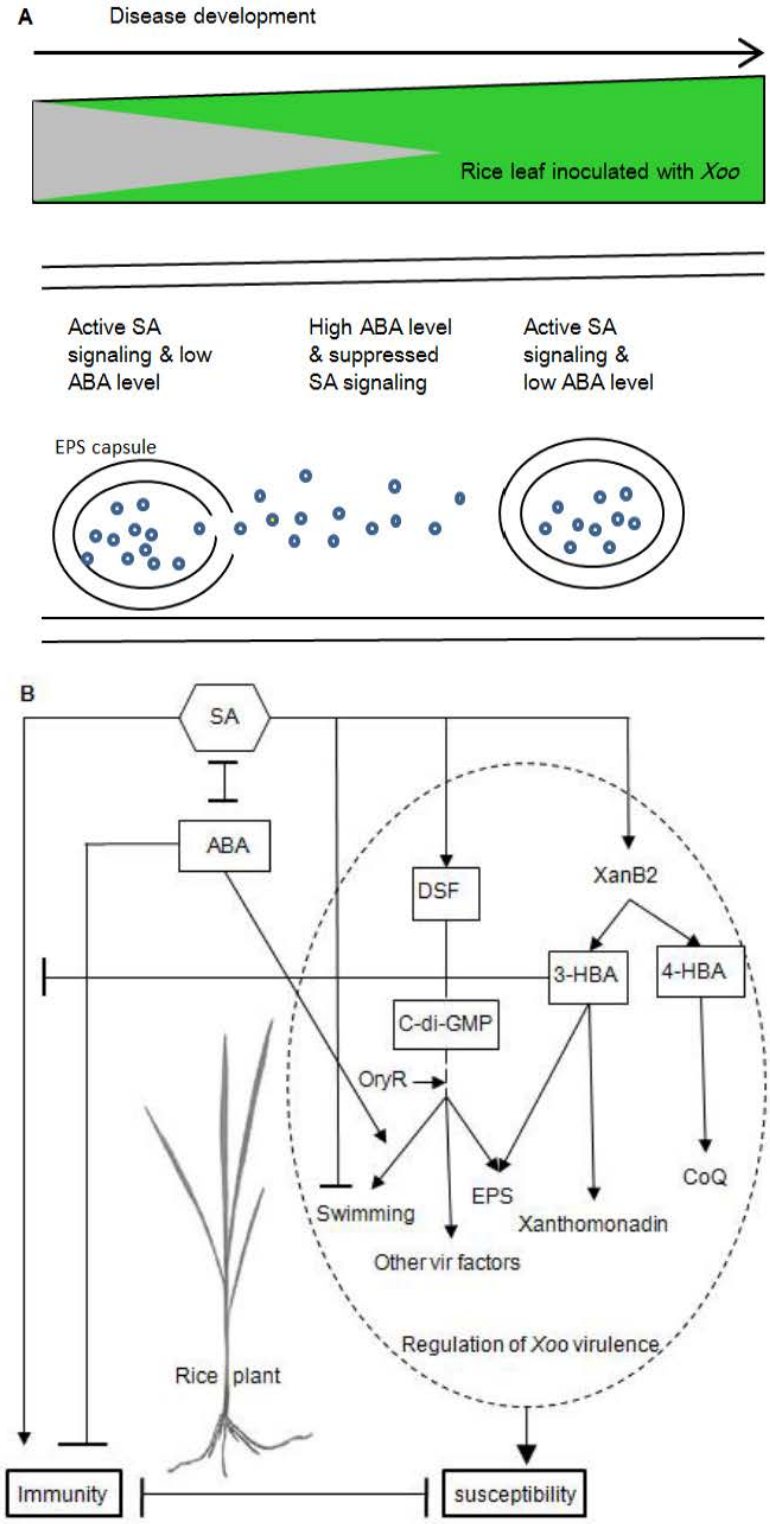

Figure 6.8 Model illustrating how crosstalk between rice SA/ABA and the DF and DSF QS systems shapes *Xoo* infection biology and molds pathological outcomes. A, The hypothetical illustration of *Xoo* infection mode inside rice leaves. B, The interplay between plant hormones and QS systems shapes the outcome of rice-*Xoo* interactions. Sharp arrows represent stimulatory effects, blunt arrows depict repressive influence.

Materials and Methods

Plant growth conditions and chemical treatments

The rice line used in this study, Taipei, was a kind gift from Dr. He (Shanghai Institute of Biological Science, China). Plants were grown in commercial potting soil (Structural, Type1) under greenhouse conditions (28 ± 4 °C, 16/8 light regimen), and fertilized with 0.5% ammonium sulphate and 0.5% iron sulphate every week.

Both DF and DSF were purchased from Sigma. DF was dissolved in distilled water, while DSF was firstly dissolved in a few drops of methanol and then diluted in distilled water. For chemical treatments, the two youngest leaves of 6-week-old plants (7- to 8-leaf stage) were detached, cut into 3-4 cm segments and floated overnight on sterile distilled water to eliminate the possible effects of wounding stress. The following day, leaf segments were floated on aqueous solutions containing various concentrations of DF or DSF and incubated for either 8 or 24 hours at 28 °C. For all experiments, leaf pieces from at least four individual plants were pooled and randomly distributed among treatments.

Bacterial strains and growth conditions

The *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) strains and plasmids used in this work are listed in Table 6.2. All strains were routinely grown on peptone sucrose agar plates (PSA; 1% peptone, 1% sucrose, 1.5% bacto agar). When necessary, antibiotics were added at the following concentrations: Ampicillin, 75 µg/ml; Kanamycin, 50 µg/ml; Gentamycin, 15 µg/ml; Tetracycline, 10 µg/ml. For RNA isolation as well as EPS bioassays, single bacterial colonies grown on PSA plates were transferred to 30 ml of peptone yeast broth (PY; 0.8% peptone, 0.2% yeast extract, 0.5% glucose, 0.2% K2HPO4, 0.05% KH2PO4, 1 mM MgSO4) containing the appropriate antibiotics and shaken at 180 rpm for 24 h at 28°C. Precultures were then diluted (OD595 = 0.08) in 50 ml PYB containing antibiotics and hormones or corresponding solvents, shaken for 24 h at 180 rpm, and subjected to the various assays described below. For EPS and xanthomonadin assays performed on PSA-grown bacteria, all cultures were grown for 4 days at 28°C, after which cells were scraped off the plates and resuspended in 15 ml sterilized water.

Table 6.2 List of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) strains and plasmids used in this study.

Strain	Characteristic	Reference
XKK12	Wild type	Ferluga *et al*. (2007)
XKK12 *OryR*$^{-}$	*oryR::Km* insertion mutant; Km^{r}	Ferluga *et al*. (2007)
XKK12 *OryR*$^{-+}$	*oryR::Km* insertion mutant harbouring the pBBR$_{Gm}$OryR plasmid; Km^{r}, Gm^{r}	Gonzalez *et al*. (2013)
XKK12 (pPIP122)	Wild type harbouring the pPIP122 plasmid; Amp^{r}, Gm^{r}	Ferluga & Venturi (2009)
XKK12 *OryR*$^{-}$(pPIP122)	*oryR::Km* insertion mutant harbouring the pPIP122 plasmid; Km^{r}, Amp^{r}, Gm^{r}	Ferluga & Venturi (2009)
XKK12 (pORYR122)	Wild type harbouring the pORYR122 plasmid; Amp^{r}, Gm^{r}	Ferluga & Venturi (2009)
XKK12 *OryR*$^{-}$(pORYR122)	*oryR::Km* insertion mutant harbouring the pORYR122 plasmid; Km^{r}, Amp^{r}, Gm^{r}	Ferluga & Venturi (2009)

Swimming assay

Xoo precultures were grown as described above and diluted to a final density of 1 x 10^{9} CFU/ml. After carefully placing 3-µl suspension droplets in the centre of soft swimming plates (0.03% peptone, 0.03% yeast extract, 0.3% agar), plates were incubated at 25°C and evaluated after either 4 or 7 days by measuring the diameter of the swimming zone.

Quantification of exopolysaccharides (EPS)

Xoo culture supernatant was collected by centrifuging at 12000 x *g* for 10 min. Afterwards, the supernatant was mixed with 1.0% potassium chloride and two volumes of absolute ethanol and incubated overnight at -20°C. After centrifuging, EPS pellets were dried for 24 h at 55°C and weighed. All values were expressed relative to the cell density.

Xanthomonadin quantification

Xanthomonadins were quantified according to the method described by Rajagopal et al. (1997). In brief, *Xoo* cells were collected from 4 ml suspension by centrifuging at 12000 x *g* for 5 min and mixed with 1 ml absolute methanol. The mixtures were then incubated for 10 min in darkness in a rotating shaker and subsequently centrifuged at 12000 x *g* for 5 min. The xanthomonadin pigments present in the supernatant were quantified by measuring OD_{445} and expressed relative to the cell density before the assay (OD_{595}).

Gene expression analysis

XKK12 cultures were grown as mentioned above and sampled when the OD595 reached 2.0. Total RNA of *Xoo* cells and rice leaves was isolated using TRIZOL (Sigma) and treated with Turbo DNase (Ambion) to remove genomic DNA contamination. First-strand cDNA was synthesized from total RNA using Multiscribe reverse transcriptase (Applied Biosystems) and random primers following the manufacturer's instructions. Quantitative PCR amplifications were conducted in optical 96-well plates with the Mx3005P real-time PCR detection system (Stratagene), using Sybr Green master mix (Fermentas) to monitor dsDNA synthesis. The expression of each gene was assayed in duplicate in a total volume of 25 mL including a passive reference dye (ROX) according to the manufacturer's instructions (Fermentas). For bacterial samples, the thermal profile used consisted of an initial denaturation step at 95 °C for 5 min, followed by 45 cycles of 95 °C for 10 sec, 60°C for 30 sec and 72°C for 30 sec. To verify amplification of one specific target cDNA, a melting-curve analysis was included according to the thermal profile suggested by the manufacturer (Stratagene). The amount of bacterial RNA in each sample was normalized using 16S rRNA (PXO_rna52) as internal control. For plant samples, we used *eEF1*α (Eukaryotic elongation factor 1alpha) as reference gene (Jain et al. 2006) and used the same thermal profile as described previously (Xu et al. 2013). All data were analyzed using Stratagene's Mx3005P software. Nucleotide sequences of all primers used are listed in Table 6.3.

β-glucuronidase gene promoter activity assays

This assay was performed as described by Ferluga and Venturi (Ferluga and Venturi 2009). In brief, 1 ml XKK12 pORY122 culture (OD595 = 2.0) was centrifuged to collect cells, which were subsequently resuspended in 600 µl GUS buffer (pH 7.0) and mixed with 23 µl 3% sodium lauryl sarcosinate and 23 µl 3% Triton X-100 in GUS buffer (10 mM EDTA, 0.1% SDS, 50 mM sodium phosphate, 0.1% Triton X-100). Following incubation for 30 min at 37 °C, 100 µl 25 mM p-nitrophenyl-β-D-glucuronic acid (pNPG) was added. The reaction was stopped by the addition of 280 µl 1 M NaCO3 after sufficient yellow color had developed. Both the optical densities at 595 nm of the Xoo cultures and the OD415 of the pNPG-treated samples were measured and 1 Miller unit of β-glucuronidase activity was defined as follows:

$$1 \text{ Miller unit} = 1{,}000 \times \{[OD415\ pNPG - (1.75 \times OD595)] / (t \times v \times OD595)\}$$

where t is the time of the reaction (in minutes), v is the volume of the culture assayed (in ml), OD_{595} is the cell density before the assay, and 1.75 is the correction factor. All measurements were performed in triplicate.

Quantification of QS factors

XKK12 cultures were grown exactly as mentioned above and sampled when the OD_{595} reached 2.0. DSF, BDSF, CDSF, DF and 4-HBA were all measured using HPLC exactly as described by (He et al. 2010; Zhou et al. 2013b)

Table 6.3 Sequences of qRT-PCR primers used in this study

Locus ID	Gene name	Forward sequence	Reverse sequence
PXO_rna52	*16sr RNA*	CCACATACTCCACCGCTTGT	ATGCGAACTGGATGTTGGGT
PXO_00068	*rpfF*	TGATGGTGTGGCAGCTCAAT	GACCGAGTTCGACTGCTTGA
PXO_00069	*rpfC*	CTGGAACCCTCCAACGTCAT	GCAATCGCTGCAACACCATA
PXO_00070	*rpfG*	GGGGTTGTCGGAAGAAGAGG	GGCGTTTCATCACGCTCATC
PXO_03945	*HDGYP*	CGAAGAAATGGACGTCATGC	TGAAATATTTTCGCCGACCA
PXO_02404	*oryR*	AGATCCAGCAGCACCAACTC	GCCGATCTCACCATTCTCGT
PXO_01397	*gumG*	AACATCCATGGCGCCCCACG	CCTGGGCGTTGGGGCTAAGC
PXO_04051	*pilA*	TCGCGCTGCCGGCTTATCAG	GGCCGCCACCGACATCCTTC
PXO_03739	*xanB2*	GCGCACATAGACTTTCAGGC	CAGCTGGAAGAAACCTTCGC
PXO_00959	*flhF*	CCTGCCAACGCCCATTTTTC	GTGATGGGCATTTGGTGGTC
PXO_01002	*flgL*	TGAACTCAGACACGCTGACC	AAGGAGTTGGCGTTGTCGAT
PXO_06158	*flgF*	TTGCATGTGGATCAGGGCTT	CGAAATACTGCCATCGCTGC
PXO_00955	*cheZ*	AAAATGCGCCACAACCTCAC	CGTCGTCTTTCTTCGGTTCG
LOC_Os03g08020.1	*eElf1a*	TTTCACTCTTGGTGTGAAGCAGAT	GACTTCCTTCACGATTTCATCGTAA
LOC_Os01g09800.1	*OsNPR1*	CACGCCTAAGCCTCGGATTA	TCAGTGAGCAGCATCCTGACTAG
LOC_Os05g25770.1	*OsWRKY45*	GGACGCAGCAATCGTCCGGG	CGGAAGTAGGCCTTTGGGTGC
LOC_Os09g25070	*OsWRKY62*	AGCTTACTTCCGCTGCGCATTC	GCGACGAATTCGGTTGTCTGCG
LOC_Os01g50700.1	*OsRab16*	CACGAGTTCAGGGATCTAGGC	AGTTGTCCATCCTCTCAAGCAA
LOC_Os02g44870.1	*OsLip9*	CGGCGGCCTCTTCGAGACAAC	TGCCAGATTGCCAGCCCGTC

Chapter 7

General discussion and future perspectives

In the absence of adaptive immunity displayed by animals, plants have evolved a wide array of constitutive and inducible defense mechanisms to resist their potential colonization by microbial pathogens and herbivorous insects. Many of these defenses are regulated by a complex network of signal transduction pathways within which plant hormones function as key signaling molecules. Although the study of plant defense signaling and pathway crosstalk is particularly relevant to plant pathology and sustainable approaches for crop production, research aimed at exploring hormonal signal transduction has been polarized to dicot model plants such as *Arabidopsis thaliana*. In contrast, very little has been done to dissect defense signaling networks in monocotyledons such as rice and other cereal crops. In view of this knowledge gap, this dissertation aimed at advancing our fundamental knowledge of the multiple hormonal signaling mechanisms underlying pathogen defense in rice. As model system, we investigated the interaction between rice and the Gram-negative leaf blight bacterium *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), one of the most important constraints on high rice productivity worldwide. Our specific objectives were threefold:

a) to decipher the tapestry of hormone signaling pathways governing susceptibility and resistance against *Xoo* and elucidate the dynamic interplay and mechanisms of crosstalk among different hormone conduits.

b) to assess if *Xoo* manipulates the rice hormone network in order to induce a state of susceptibility and elucidate the underlying mechanisms

c) to investigate whether and how plant hormones are involved in interkingdom signaling and affect microbial virulence by interfering with bacterial cell-to-cell communication.

7.1 Main research findings and practical implications

7.1.1 ABA, CK and SA: interactive protagonists in rice-*Xoo* interactions

Although our knowledge of the rice hormone signaling circuitry controlling resistance and susceptibility against *Xoo* is still limited, evidence is accumulating that SA, JA and BR enhance disease resistance (Yuan et al. 2007; Tao et al. 2009; Yamada et al. 2012; Nakashita et al. 2003), whereas auxin, ET and GA promote susceptibility (Fu et al. 2011; Shen et al. 2011; Yang et al. 2008). In adding to this list, this study uncovered ABA and CK as two additional players determining the outcome of rice-*Xoo* interactions. By combining exogenous hormone applications, chemical inhibition of de *novo* hormone synthesis and genetic disruption of plant hormone signaling, we found that activation of ABA biosynthesis and ABA signaling tightly correlates with BLB development (Chapter 4). In addition, exogenously

administered ABA strongly promoted *Xoo* growth *in planta*, suggesting that ABA functions as a virulence factor for the bacterium (Chapter 4). Similarly, our data also pinpointed a negative role for CK in *Xoo* resistance (Chapter 5).

Of particular note, bioassays with different rice lines revealed that ABA and CK were only effective in susceptible interactions but not in rice genotypes carrying *Xa13*, a major resistance (*R*) gene conferring high levels of resistance to *Xoo*. Although this suggests that both hormones may predominantly affect basal immune responses in rice (Chapter 4 and Chapter 5), care should be taken when interpreting these results. In Arabidopsis, for instance, ABA is known to antagonize ETI mounted by the resistance proteins SNC1 and RPS4 (Mang et al. 2012). Likewise, Jiang et al. (2010) previously demonstrated the ability of ABA to suppress *R* gene-mediated resistance to *M. oryzae* (Jiang et al. 2013). Therefore, we cannot rule out the possibility that ABA and CK may affect ETI governed by *R* genes other than *xa13*.

Previously, Argueso et al (2013) showed that, depending on the relative hormone concentration *in planta*, CK can play both positive and negative roles in defense of Arabidopsis against the biotrophic oomycete *Hyaloperonospora arabidopsidis*. High CK concentrations increased SA-mediated resistance, whereas low concentrations of CK rendered plants hyper-susceptible (Argueso et al. 2012). Strikingly different results, however, were obtained in response to the rice blast pathogen *M. oryzae*. In this interaction, pretreatment with CK had no significant impact on disease susceptibility, whereas higher doses increased blast incidence (Jiang et al. 2013). Conversely, we found both low and high concentrations of CK to increase susceptibility to *Xoo* (Chapter 5). Together these findings indicate that CK signaling can cascade to either the benefit or the detriment of the plant. Similar to what has been observed for other 'growth hormones' such as BRs and GAs (De Bruyne et al. 2014), it thus seems that CK plays ambiguous roles in the plant defense signaling network, the effect of which cannot easily be generalized.

SA has long been considered to be one of the most important defense hormones and, accordingly, many other hormones have been found to intimately interact with SA. For instance, Jiang et al (2010) reported that SA enhances resistance against the rice blast fungus *M. oryzae* at least in part by counteracting ABA induced-susceptibility (Jiang et al. 2010). In a similar vein, we found reciprocal ABA-SA antagonism to play an important role in determining the outcome of rice-*Xoo* interactions (Chapter 4). Using various SA signaling mutants, we showed that ABA suppresses SA signaling downstream of *OsWRKY13* but upstream of or at the level of *OsNPR1* and *OsWRKY45* (Chapter 4). In contrast, we found little evidence of CK-SA signal interactions, neither in non-stressed rice plants nor in leaves responding to *Xoo* infection (Chapter 5).

Unlike the situation in Arabidopsis where NPR1 controls almost all SA-responsive genes, the

rice SA pathway branches into two sub-pathways controlled by *OsNPR1* and *OsWRKY45*, respectively (Liu et al. 2007; Qiu et al. 2008; Tao et al. 2009). Notably, constitutive expression of *AtNPR1* in rice leads to enhanced resistance against a wide range of fungal and bacterial pathogens but confers susceptibility to viral and abitotic stresses (Quilis et al. 2008). In accordance, overexpression of *OsNPR1* not only confers substantial resistance to *Mo* and *Xoo*, but also leads to increased sensitivity to light and hypersusceptibility to herbivorous insects, which might hamper the application of *OsNPR1* OX plants in the field (Chern et al. 2005; Yuan et al. 2007; Sugano et al. 2010). Interestingly, constitutive localization of site-mutated OsNPR1 in the nucleus abolishes the herbivore susceptibility associated with OsNPR1-conditioned pathogen resistance, a phenomenon which is most likely due to de-repression of JA signaling under these conditions (Yuan et al. 2007). These studies elegantly illustrate the importance of OsNPR1 in regulating and intertwining various hormone pathways and underscore the potential of site-mutated OsNPR1 as a target for engineering broad-spectrum stress tolerance in rice.

Another promising target for rice improvement is the ABA-inducible mitogen-activated protein kinase OsMAPK5. Our data suggest that OsMAPK5 is vital for ABA-induced susceptibility towards *Xoo* (Chapter 4). Interestingly, OsMAPK5 also is a pivotal player in the ABA-induced resistance against *C. miyabeanus*, and in basal susceptibility of rice towards *M. oryzae*, *B. glumae* and *H. oryzae* (De Vleesschauwer et al., 2010; Nahar et al., 2012; Xiong & Yang, 2003). On the other hand, *OsMAPK5* RNAi plants are more susceptible to drought, salt, and cold treatments (Xiong and Yang 2003), while recent findings also implicate OsMAPK5 in JA-mediated defense against chewing herbivores (Wang et al. 2013). Emerging from these findings is the view that OsMAPK5 is positioned at the intersection of various hormone and stress signaling pathways, acting as a key hub for pathway crosstalk and signal crosstalk. Considering the complexity of its regulation, It will therefore be important not to constitutively alter the expression of *OsMAPK5* to engineer disease-resistant plants, but for instance to use pathogen- or stress-inducible promoters in order to fine-tune the transcription of OsMAPK5 to the type of stress encountered.

Giving the significance of ABA and SA in promoting or suppressing a variety of rice diseases, respectively, timely application of ABA inhibitors and/or SA elicitors may provide an attractive tool for efficient disease control in the field. Supporting this notion, combinations of abamine, a highly specific ABA-biosynthesis inhibitor (Han et al. 2004), and BTH or BIT (benzisothiazole), two elicitors of the SA pathway, were shown to remarkably increase the efficiency of rice blast control and reduce the amount of chemical inducers required to prevent the disease (Yoshida et al. 2006). Effective disease management has also been obtained with probenazole, a chemical that is widely used in Japan and primes rice for enhanced resistance against a variety of pathogens, including *M. oryzae*, *Xoo*, *C. miyabeanus* and *B. glumae* (Iwata et al.

2004). Since chemical defense inducers have no direct effect on pathogens, they are less likely to lead to resistance-buildup, a side effect that is often problematic for pesticides. Moreover, given the environmentally friendly nature and broad-spectrum effectiveness of these so-called plant activators, there is considerable commercial interest in this area.

7.1.2 Role of growth-defense trade-offs in *Xoo* resistance

While the deployment of defense mechanisms is imperative for plant survival, defense activation generally comes at the expense of plant growth (Figure 7.1). This growth versus defense conflict is based on the premise that plants possess a limited pool of resources, which demand prioritization towards either growth or defense depending on external and internal factors (Huot et al. 2014). As plants must both grow and defend in order to survive and reproduce, growth-defense trade-offs have important ecological, agricultural and economic consequences (Walling 2008; Baldwin et al. 2001).

In common with findings in other pathosystems, numerous studies have suggested a trade-off between plant growth and development and defense to *Xoo*. For instance, both *OsNPR1* overexpressing and CK-deficient *Gn1a* plants not only display increased levels of BLB resistance but also exhibit varying degrees of semi-dwarfism (Chapters 4 and 5). Similarly, *Xoo*-resistant rice lines overexpressing *OsWRKY45* under the control of a strong constitutive promoter showed strong growth defects in field trials (Takatsuji and Jiang 2014). Intriguingly, our findings suggest that *Xoo* may exploit these growth and defense trade-offs as a virulence strategy. This notion was borne by the observation that CK promotes BLB susceptibility by activating TOR and degrading SnRK1, two ancient growth-regulatory proteins. In yeast and mammals, SnRK1 safeguards cellular homeostasis by promoting catabolism and repressing anabolism, whereas TOR constitutes a master switch for activation of cell growth under nutrient-rich conditions (Zoncu et al., 2011). Accumulating evidence indicates that TOR and SnRK1 fulfill similar roles in plants (Ghillebert et al. 2011; Xiong and Sheen 2014). By exploiting the positive effect of CK on TOR activity, *Xoo* may thus tilt the growth-vs-defense balance towards growth, thereby reducing resources available for the plant to mount an effective defense response. In support of this assumption, Szczesny et al. (2010) reported that AvrBsT, a YopJ family effector of the pepper pathogen *Xanthomonas campestris* pv. *vesicatoria*, interacts with SnRK1 to suppress the hypersensitive response that is elicited by the AvrBs1 effector (Szczesny et al. 2010). Furthermore, the Arabidopsis SnRK2 phosphorylates a type III effector HopQ1 from *Pseudomonas syringae* pv. *phaseolicola* (Giska et al. 2013). These data suggest that plant pathogens may have evolved sophisticated strategies to evade or suppress SnRK1 action in order to strengthen their virulence. Moreover, like CKs, other plant growth-promoting hormones such as auxin and GA are likely to activate

TOR signaling (Bögre et al. 2013; Miyamoto et al. 2012). Therefore, in addition to suppressing SA and/or JA responses, these susceptibility-promoting hormones may antagonize host immunity by disrupting SnRK1-mediated defense through activation of antagonistic TOR signaling.

Understanding the molecular processes governing plant prioritization and diversion of resources towards growth or defense will provide powerful tools to genetically tailor plants that optimize this balance to maximize crop yield under variable environmental conditions. In this context, our recent observation that double transgenic plants overexpressing TOR and the PAMP receptor protein Xa21 showed both strong *Xoo* resistance and an almost twofold increase in seed yield compared to wild-type is of particular interest (De Vleesschauwer D, in preparation). While the underlying molecular processes are still poorly understood, these findings may provide a proof-of-concept, illustrating how growth-defense trade-offs can be circumvented by fine-tuning the negative impact of TOR on PTI signaling. Likewise, targeted overexpression of SnRK1 under control of a tightly regulated and stress-inducible promoter might be an elegant solution to break the link between enhanced stress tolerance and growth restriction.

7.1.3 Rice-*Xoo* interkingdom signaling occurs through plant hormones and bacterial QS factors

Most research aimed at deciphering the role of phytohormone signaling networks in the regulation of plant-microbe interactions is focused on exploring how hormones activate or suppress innate immune responses in plants. In contrast, surprisingly little is known about whether hormones also directly influence the virulence machinery of plant pathogens. In their seminal review, Bauer and Mathesius (2004) proposed that bacteria might use the hormonal signals of eukaryotic hosts as cues to activate virulence gene expression needed for infection of that host (Bauer and Mathesius 2004). However, despite some recent progress, relatively little advances have been made over the past decade and much remains to be learned about the mechanism(s) and functional importance of such host-pathogen interkingdom signaling. In Chapter 6, we showed that ABA promotes the swimming motility of *Xoo* whereas SA exerts the opposite effect. Interestingly, by stimulating the biosynthesis of the QS signals DSF, DF (3-HBA) and 4-HBA, SA also triggers activation of multiple bacterial virulence traits including EPS, xanthomonadin pigments, and most likely CoQ (Chapter 6). EPS and xanthomonadin are important virulence factors that protect xanthomonads from abiotic stresses encountered during epiphytic growth, as well as from toxic molecules encountered during growth in plant tissue (Büttner and Bonas 2010). CoQ is a key cofactor in the aerobic respiratory electron transfer for energy generation and protects bacteria from peroxidative damage (Cluis et al.

2007). In view of these findings, it is tempting to speculate that despite functioning as a positive regulator of rice immunity by inducing plant defense responses and restricting the motility of *Xoo*, SA also ended up being exploited by *Xoo* as a signal to activate its QS-mediated virulence machinery that is needed for plant infection. Intriguingly, Sperandio et al. (2003) reported that enterohemorrhagic *Escherichia coli* (EHEC serotype O157:H7) uses the mammalian hormone epinephrine as an alternative QS signal in order to 'sense' that it is within the intestine and to coordinate the expression of virulence genes. These remarkable similarities in utilizing host hormones as signals to activate QS signaling or strengthen QS-dependent virulence traits during host infection by plant and mammalian pathogens strongly suggest that QS-mediated interkingdom signaling is an evolutionally conserved virulence strategy of pathogenic bacteria.

In contrast with our findings in *Xoo*, SA was previously shown to interfere with bacterial communication, a process generally known as "quorum quenching". Yuan et al. (2007b) reported that SA induces the expression of a lactonase which degrades AHL-type QS signals, consequently shutting down the expression of *vir* regulon in *Agrobacterium tumefaciens*. Similarly, Zhang and his colleagues (2001) identified an AHL-degrading enzyme from *Bacillus* designated AiiA (autoinducer inactivation) that is able to disrupt QS communication and lower the virulence of pathogenic bacteria. Introducing this gene into tobacco and tomato plants led to dramatically enhanced resistance towards the soft rot pathogen *Erwinia carotovora* (Dong et al. 2001). These findings shed light on a new direction for disease control, either by introducing genes that encode quorum quenching enzymes into plants or by recruiting the microbes which produces the desirable quorum quenching enzymes as biocontrol agents (Molina et al. 2003; Dong et al. 2000).

However, as listed in a recent review by Chen et al (2013), all quorum quenching enzymes characterized to date are specific for AHL-type signals whereas none have been found to be effective against DSF signals. Interestingly, recent studies on DSF signaling mechanisms in *Xanthomonas* spp. suggest that RpfC, in addition to perceiving DSF signals, also negatively regulate DSF biosynthesis via direct protein-protein binding with RpfF (Cheng et al. 2010). Giving the strong loss of virulence seen in DSF-deficient *Xoo* mutants (Chatterjee and Sonti 2002; Jeong et al. 2008), application of DSF-inactivating chemicals or alternative compounds that block DSF-mediated QS systems by either antagonizing DSF perception or by interfering with DSF synthesis and/or signaling (e.g. by interfering with RpfF/C), seem to be promising strategies for efficient and robust disease control in the field.

Belonging to a family of LuxR proteins that have the same modular structure as QS LuxRs but are devoid of a cognate LuxI AHL synthase, the so-called LuxR 'solo' OryR controls the expression of more than 300 bacterial genes and is important for the complete virulence of *Xoo* (Ferluga et al. 2007; Gonzalez et al. 2013). Unlike other LuxR solos, OryR does not

respond to a wide range of AHL signals. Instead, it interacts with an unknown rice signal molecule (RSM) that is present in the xylem of rice leaves (Ferluga et al. 2007; Ferluga and Venturi 2009). Accordingly, Gonzalez et al. (2012, 2013) reported that OryR regulates virulence gene expression only when rice macerate is present in the culture medium (Gonzalez et al. 2012; Gonzalez and Venturi 2013). Interestingly, our work revealed that OryR is required for induction of EPS synthesis by SA and obligatory for ABA-promoted swimming. Moreover, SA strongly induced *oryR* transcription and activated *oryR* promoter activity in GUS reporter assays. One possible interpretation of these findings is that *Xoo* uses OryR to "eavesdrop" on rice SA, and possibly also ABA, titers. Whichever mechanism operative, it is clear that OryR plays a prominent role in regulating rice-*Xoo* signal interactions. Future studies should be focused on exploring the precise mechanisms by which OryR coordinates regulation of *Xoo* QS circuits by ABA/SA.

7.1.4 The hormone signature of Xoo-inoculated rice: distinguishing friends from foes

Despite the extensive body of literature demonstrating the importance of phytohormones in plant immunity, our knowledge about how hormone levels change throughout the course of an infection is still limited. Most studies addressing the impact of phytohormones on pathogenicity consider individual hormones in isolation, and often infer a dominant hormone influence from endpoint measurements of a limited set of hormone-specific markers, thus missing changes in hormone dynamics that underlie the final phenotype. However, mounting evidence suggests that single hormone measurements cannot accurately reflect the plant's physiological state because of the complex crosstalk among individual hormone pathways. A further layer of complexity arises from the often multifaceted role of hormones in disease and resistance, and their spatial biosynthesis in relation to the invading pathogens.

Therefore, to capture the spatio-temporal specificity that is central to fine-tuning hormone signaling and responses, highly sensitive analytical methods enabling the simultaneous detection and quantification of multiple phytohormones are needed.

Over the past few years, several reports have been published outlining multi-component hormone measurements. In collaboration with the Sakakibari lab (RIKEN, Japan), we have used ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) to profile the hormone signature of *Xoo*-infected rice leaves. Samples were taken at 1, 2, 4 and 8 days after inoculation with the virulent *Xoo* strain PXO99 and subjected to a highly sensitive and high-throughput detection method enabling the simultaneous analysis of 43 molecular species of CKs, auxins, ABA, SA, JA and GAs.

As shown in Table 7.1 and consistent with our data presented in Chapter 4, quantification of endogenous ABA content showed that ABA levels strongly increased throughout the course of infection (Chapter 4). In conjunction with the extensive transcriptional reprogramming of ABA-responsive genes in *Xoo*-infected leaves and the *Xoo* resistance-inducing effect of the ABA biosynthesis inhibitors fluridone (Chapter 5) and abamine (Yoshioda et al., 2006), these findings strongly infer that *Xoo* co-opts the rice ABA pathway to cause disease. Such manipulation of the plant ABA machinery has already been observed in a number of other pathosystems, including the Arabidopsis – *Pseudomonas syringae* pv. *tomato* (*Pst*) interaction (De Torres Zabala et al. 2009; Grant et al. 2007). Like many other pathogens, *Pst* hijacks the Arabidopsis ABA machinery via hereto-specialized effector proteins such as HopAM1 and AvrPtoB (Goel et al. 2008; Grant et al. 2007). Similarly, a recent report showed that ectopic expression of the AvrXccC effector protein of *Xcc* triggers ABA accumulation in transgenic Arabidopsis by inducing the ABA biosynthesis gene *NCED5* (Ho et al. 2013). Considering the ability of ABA to interfere at multiple levels with various stress-response pathways (Asselbergh et al. 2008), manipulating host ABA signaling and hijacking ABA crosstalk mechanism likely represents a powerful virulence strategy, controlling the outcome of numerous plant-pathogen interactions.

Besides eliciting ABA synthesis, *Xoo* infection also induced the accumulation of several bioactive CK species and their corresponding precursors at 8 dpi. Surprisingly, however, the levels of inactive CKs remained fairly static following infection, suggesting that *Xoo* specifically stimulates *de novo* synthesis of CK, but not the inactivation nor the degradation steps (Chapter 5). Moreover, considering that the *Xoo* genome harbors a close homolog of the CK-biosynthesis gene *isopentenyl transferase,* it is tempting to speculate that the high CK levels observed in PXO99-inoculated plants are at least partly of microbial origin.

A series of recent studies by Shiping Wang's group demonstrated that auxin negatively regulates resistance to *Xoo*, with both exogenous IAA, infection-induced plant IAA and *Xoo*-secreted IAA promoting susceptibility (Ding et al. 2008; Fu et al. 2011). In line with these findings, our hormone quantification data showed that IAA levels are significantly boosted by virulent *Xoo* infection, further suggesting that IAA in susceptible plants acts as a bacterial virulence factor (Table 7.1). Liu et al. (2012) previously reported that ABA and IAA show similar accumulation patterns in response to *Xoo* infection. With accumulating evidence pointing towards ABA-IAA crosstalk in regulating plant growth and development (Belin et al. 2009), it will be worth analyzing whether the two hormones function synergistically in the rice-*Xoo* interaction.

Much like ABA, CK and IAA, *Xoo* infection also induced the accumulation of several GAs including GA_8 and GA_{19}. Although GA has only recently been linked to plant immunity, several reports have suggested a negative role of the hormone in rice defense against *Xoo* (Qin et al.

2013; Yang et al. 2008). Analysis of the mechanisms of action suggested that GA favors *Xoo* infection by inducing the degradation of SLR1, the only DELLA protein in rice. In agreement with SA and JA positively regulating *Xoo* resistance, SLR1 serves as a main target of SA and JA-mediated growth inhibition and is required for expression of SA- and JA-responsive genes (De Vleesschauwer et al., unpublished).

Contrary to dicot plants where basal SA levels are low but strongly rise following infection, rice accumulates high basal levels of SA that are only weakly responsive to pathogen attack (Silverman et al. 1995; Chapter 2). Consistent with these data and corroborating the relatively weak effect of *Xoo* on the expression of SA marker genes (*OsWRKY45*, *OsNPR1* and *OsWRKY13*), we also found SA levels to barely change upon *Xoo* infection, except for a late, twofold increase at 8 dpi (Chapter 4). Analysis of the SA kinetics in incompatible rice-*Xoo* interactions revealed a fairly similar accumulation pattern (Liu et al. 2012). Together with the finding that SA-deficient *NahG* rice plants show unaltered expression of SA-responsive *PR* gene expression (Xiong and Yang 2003) and considering the strong levels of disease resistance obtained in plants overexpressing *OsNPR1* and *OsWRKY45* (Shimono et al. 2007; Yuan et al. 2007), these findings support the idea that the signaling action of SA, rather than *de novo* biosynthesis, is important for *Xoo* resistance. In contrast, levels of JA strongly responded to *Xoo* infection and peaked at 8 dpi at approximately 20 times the levels found in non-inoculated controls (Table 7.1). Rice lines resistant to *Xoo* are reported to accumulate even higher levels of JA upon pathogen attack (Liu et al. 2012), further suggesting that the high JA titers in PXO99-infected leaves are associated with resistance, rather than susceptibility.

Though preliminary in nature, our hormone profiling approach offers a first insight into the complex hormone dynamics governing rice immunity against *Xoo* and the intervention strategies used by the pathogen to hijack, evade or suppress hormone signaling and crosstalk. In addition to triggering *de novo* synthesis of immunity-associated JA and setting off SA-regulated defense signaling, virulent *Xoo* infection also induces plant biosynthesis of ABA, CK, auxin and GA, all of which are known to favor pathogen invasion and replication. Considering the ability of *Xoo* to produce IAA and possibly CK itself, and given the ability of bacterial QS factors to inhibit SA-responsive gene expression (Chapter 6), it thus seems that rice and *Xoo* engage in a fierce and continuous battle for dominance over multiple hormone pathways (Figure 7.1).

Table 7.1 Concentrations (pmol/g FW) of plant hormones in T65 leaves after infection with virulent *Xoo* strain PXO99.

dpi	0	1		2		4		8	
	Mock	Mock	Infected	Mock	Infected	Mock	Infected	Mock	Infected
ABA	187.99±15.98	334.38±68.08	355.4±7.99	215.88±23.32	260.25±22.52	197.11±31.8	398.08±90.25	405.67±51.78	8074.42±821.9
tZR	3.27±ND	0.47±ND	0.75±ND	0.63±0.13	0.71±0.2	0.68±0.13	0.74±0.19	1.04±ND	4.27±1.89
tZRPs	4.31±ND	2.11±ND	3.55±ND	1.07±0.2	1.09±0.34	1.95±0.6	1.7±0.04	1.91±ND	12.31±3.51
cZ	2.22±ND	5.82±ND	21.23±ND	2.51±1.24	26.66±11.55	14.09±8.65	17.87±3.08	8.29±ND	24.41±3.16
cZR	1.55±ND	1.6±ND	2.44±ND	1.03±0.12	3.09±1.45	3.14±0.91	3.77±1.41	10.6±ND	35.46±7.45
cZRPs	0.55±ND	0.67±ND	1.95±ND	0.55±0.11	2.4±0.91	1.32±0.67	1.74±0.34	1.56±ND	22.66±11.56
DZ	0.11±ND	0.25±ND	1.16±ND	0.29±ND	1.51±0.5	0.66±0.4	1.12±ND	0.34±ND	2.27±ND
iP	0.56±ND	0.15±ND	0.36±ND	0.19±0.04	1.35±2.01	1.01±0.5	2.87±3.63	0.78±ND	6.6±6.55
iPR	0.51±ND	0.11±ND	0.28±ND	0.1±0.02	0.49±0.5	0.6±0.41	1.13±0.79	1.14±ND	21.07±7.39
iPRPs	5.52±ND	1.75±ND	3.32±ND	2.09±0.31	4.49±4.09	5.76±2.28	11.27±7.63	8.04±ND	40.54±7.35
tZ9G	245.64±ND	104.94±ND	114.2±ND	139.22±33.62	134.51±43.19	126.92±9.89	113.18±48.17	116.45±ND	74.35±10.87
tZOG	4.44±ND	4.66±ND	5.46±ND	4.56±0.55	4.04±0.71	3.83±0.88	4.27±0.26	3.16±ND	2.35±0.59
cZOG	1357.29±ND	1564.81±ND	1691.62±ND	1499.77±70.78	1414.59±204.9	1370.93±92.65	1637.09±165.57	874.68±ND	1002.31±86.83
cZROG	32.31±ND	15.68±ND	19.24±ND	15.47±0.78	19.28±5.7	24.66±3.78	28.7±15.13	32.76±ND	25.29±2.11
cZRPsOG	1.63±ND	1.86±ND	3.57±ND	1.59±0.14	2.73±0.62	2.91±0.94	2.55±0.26	1.85±ND	3.11±0.95
DZ9G	3.32±ND	2.02±ND	1.86±ND	1.79±0.11	1.79±0.32	2.27±0.34	2.13±0.18	2.37±ND	1.57±0.47

Continued

dpi	0	1		2		4		8	
	Mock	Mock	Infected	Mock	Infected	Mock	Infected	Mock	Infected
iP9G	7.16±ND	4.02±ND	4.62±ND	4.44±2.27	8.12±7.69	8.41±1.78	7.98±1.21	6.32±ND	8.29±3.17
IAA	89.55±14.16	86.14±14.35	97.93±19.82	83.53±13.47	113.06±14.81	136.91±16.94	205.24±89.01	317.36±106.16	755.76±276.71
IAAsp	50±29.71	10.4±7.65	15.8±2.39	15.44±4.57	28.36±4.44	99.35±45.95	91.06±33.35	144.26±69.78	170.81±23.68
GA4	24.21±ND	4.8±ND	10.72±ND	13.87±ND	11.54±ND	38.5±29.22	31.44±ND	16.13±ND	0±ND
GA7	0±ND	0.41±ND	1.15±ND	4.49±ND	0±ND	2.38±ND	1.53±ND	0±ND	0±ND
GA8	0±ND	3.32±0.59	3.84±0.5	2.3±0.49	3.2±0.7	3.44±0.54	3.81±0.73	4.34±0.57	9.02±2.21
GA9	5.64±1.77	3.41±1.1	4.07±1.39	5.09±2.37	7.49±2.25	30.82±22.13	30.81±41.54	10.82±1.36	11.53±5.32
GA19	57.21±42.4	45.71±15.28	49.31±7.46	32.93±2.73	55.14±8.46	39.43±3.83	79.72±29.07	63.56±23.17	211.73±128.42
GA20	6.62±0.29	4.43±0.97	4.8±0.79	5.26±1.46	4.8±0.98	5.1±0.26	7.75±2.59	8.57±1.06	14.21±7.56
GA24	0.68±0.47	0.25±ND	0.57±0.29	0.53±0.32	0.4±0.11	0.76±0.55	0.62±0.35	0.16±0.06	0.28±ND
GA44	5.46±2.72	2.12±0.52	3.04±1.13	2.58±0.85	4.17±0.38	3.94±0.79	5.33±0.89	8.09±4.57	3.68±0.19
SA	341640.85 ±145719.97	360323.62 ±29645.48	349830.16 ±34267.49	357241.73 ±41476.82	376594.63 ±19487.22	425430.7 ±41222.79	385440.59 ±33149.33	389515.78 ±102760.38	613235± 12043.41
JA	173.98±222.59	449.54±100.09	409.1±70.41	89.51±27.24	172.43±7.47	114.88±28.3	116.13±31.54	151.49±101.85	9472.83±2694.18

Data presented are mean ± SD of three biological repetitions. tZR, trans zeatin riboside; tZRP, tZR phosphates; cZ, cis zeatin; cZR, cZ riboside; cZRP, cZR phosphates; DZ, dihydrozeatin; iP, N6-(Δ2-isopentenyl) adenine; iPR, iP riboside; iPRP, iPR phosphates; tZ9G, tZ 9-glucoside; tZOG, tZ O-glucoside; cZOG, cZ O-glucoside; cZROG, cZR O-glucoside; cZRPsOG, cZPs O-glucoside; DZ9G, DZ 9-glucoside; iP9G, iP 9-glucoside; IAAsp: IAA-aspartic acid. ND: not determined because there were only two samples. dpi: days post inoculation.

Main conclusions

In summary, the work presented in this dissertation has addressed most if not all of the original research questions and has led to the following conclusions:

1. ABA plays a dual role in promoting BLB development by promoting swimming motility of *Xoo*, on the one hand, and antagonizing SA-mediated defenses in rice, on the other.

2. CK similarly acts as a virulence factor of *Xoo*. However, rather than directly interacting with the SA pathway or acting as a bacterial QS agonist, CK seems to condition susceptibility to *Xoo* by tricking the plant into activating a central growth-promoting pathway controlled by the master regulatory protein Target of Rapamycin (TOR).

3. Despite its essential defensive role in the rice immune signaling network and its ability to restrict bacterial swimming motility, SA is also exploited by *Xoo* as an alternative QS signal. Reversely, the *Xoo* QS signal DF likely functions as an SA mimic to disturb SA-mediated defense in rice.

4. The LuxR solo protein OryR is a pivotal regulator of rice-*Xoo* interkingdom signaling. Being indispensable for SA-induced EPS production and ABA-promoted swimming and transcriptionally activated by SA, we speculate that *Xoo* employs OryR to ‘eavesdrop’ on rice SA and ABA titers.

5. *Xoo* infection boosts *de novo* synthesis of ABA, bioactive CKs, auxin and several GAs, which in turn favor *Xoo* infection. Meanwhile, *Xoo* also triggers production of the rice defense hormones SA and JA, suggesting that both plant and attacker try to differentially engage distinct hormone pathways within defined temporal windows.

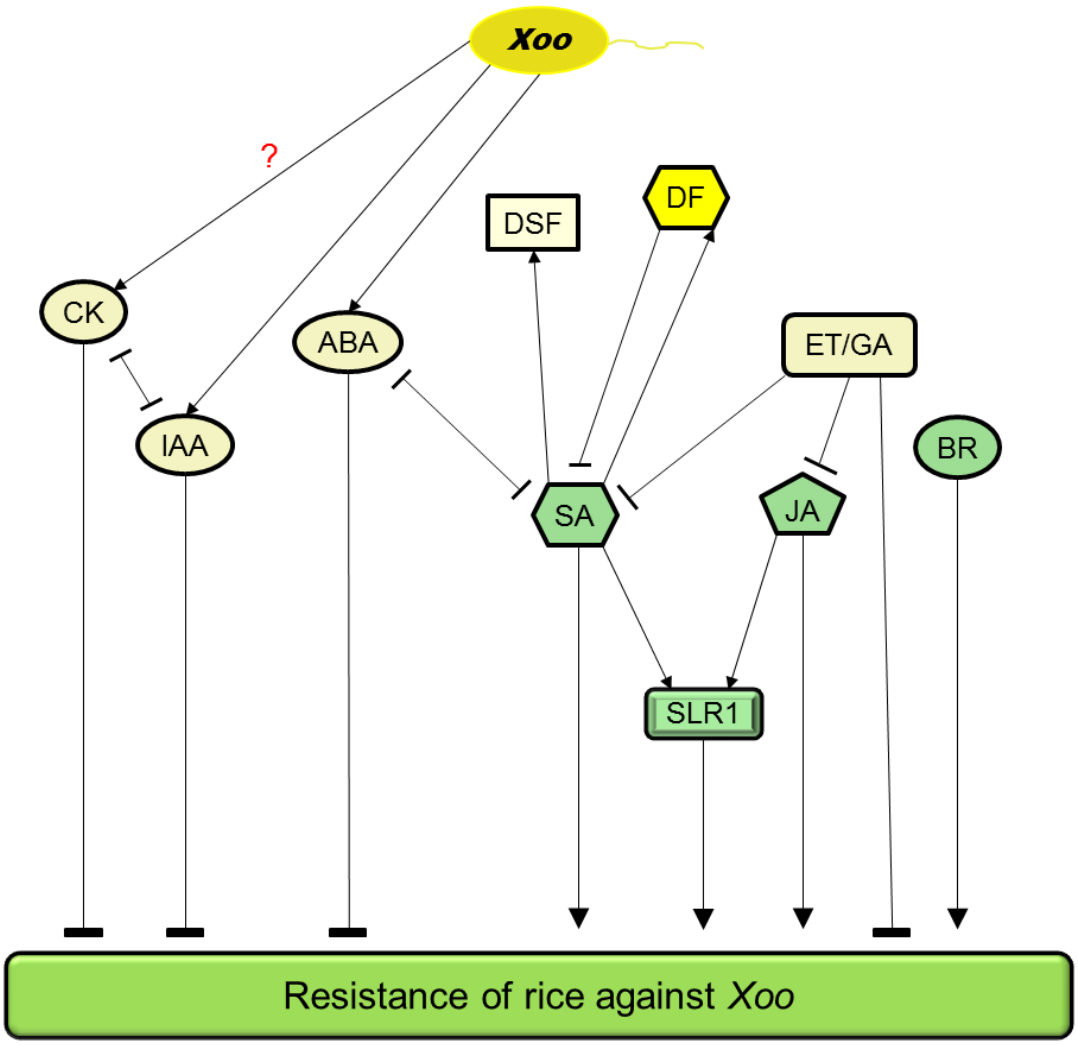

Figure 7.1. Schematic illustrating the dynamic interplay among plant hormones and *Xoo* QS signals in shaping the resistance of rice against *Xoo*. Sharp arrows represent stimulatory effects and blunt arrows represent repressive effects. ?: need more evidence.

7.2 Future perspectives

Although the results denoted in this dissertation have significantly advanced our understanding of hormone defense networking in rice and rice-*Xoo* interkingdom signaling in particular, several aspects deserve further experimental investigation. In addition, this work has opened up several perspectives for future studies.

- We revealed that ABA and CK negatively regulate defense of rice against *Xoo* (Chapter 4 and Chapter 5). In addition, we assessed their interactions with the defense hormone SA and evaluated the importance of such hormone crosstalk in determining the outcome of rice-*Xoo* interaction. However, whether ABA and CK also interact with other hormones in facilitating *Xoo* invasion remains unclear. For instance, exciting new findings revealed a positive correlation between ABA and auxin during abiotic stress (Du et al. 2012). Giving that both ABA and auxin are virulence factors to *Xoo*, it is of particular interest to investigate whether they also work synergistically to promote BLB susceptibility. The BR biosynthesis, regulation and signaling genes are all up-regulated in transgenic rice plants expressing a bacterial *IPT* gene under control of a stress-inducible promoter (P_{SARK}::*IPT*), suggesting that BR and CK act in concert (Peleg et al. 2011). In contrast, JA biosynthesis and signaling genes are downregulated in response to water stress in P_{SARK}::*IPT* plants compared to the WT, which raises the prospect of negative crosstalk in the direction of CK damping JA action (Peleg et al. 2011). Considering that JA positively regulates *Xoo* resistance, it is not inconceivable that antagonistic crosstalk between CK and JA may contribute to CK-mediated susceptibility.

  Moreover, identification and characterization of key 'hubs' that connect different signaling pathways are important for potential practical use. A promising candidate is SLR1, the only DELLA protein in rice and a central repressor of GA signaling. Positioned at the interface of stress and developmental signaling, DELLA proteins crosstalk with multiple hormone pathways, allowing appropriate modulation of plant growth and defense in response to various stimuli (De Bruyne et al., 2014). In

Arabidopsis, they were shown to differentially affect defense responses to biotrophic and necrotrophic pathogens by modulating JA and SA signaling (Navarro et al. 2008). In rice, SLR1 serves as a main target of JA-mediated growth inhibition (Yang et al. 2012a). Recently, our group reported that SLR1 plays a key role in resistance of rice against the root pathogen *P. graminicola* by interfering with BR signaling (De Vleesschauwer et al. 2012). Moreover, mounting evidence suggests that SLR1 coordinates and adjusts interplay between environmental and hormone signals to shape immunity of rice against hemibiotrophic pathogens (De Bruyne et al., 2014). Apart from its importance in plant growth (Claeys et al. 2014; Ikeda et al. 2001), these findings thus favor a central role for SLR1 in fine-tuning rice immunity by integrating and coordinating multiple hormone cascades.

- As illustrated in Chapter 5, CK-induced susceptibility is at least partially due to its synergistic and antagonistic effect on TOR and SnRK, respectively. However, little is known about the interaction between TOR/SnRK and other hormones and the possible significance of these signal interaction in rice resistance and susceptibility. Considering that auxin and GA have recently been shown to activate TOR signaling in Arabidopsis (Schepetelnikov et al., 2013; Miyamoto et al. 2012) and given the ability of ABA to degrade SnRK1 in wheat (Coello et al. 2012), this may be a fruitful area for future research.
- In Chapter 6, we demonstrated that the plant hormones ABA and SA directly affect *Xoo* virulence through their effect on the bacterial QS machinery. Yet, the functions of other plant hormones were left for future investigation. Potentially interesting candidates are CK and auxin, both of which augment BLB susceptibility in rice and are likely produced by both plant and pathogen. Furthermore, how *Xoo* perceives and processes plant hormone signals is still obscure and requires more investigation. Moreover, as we mentioned in the general discussion section, host hormones modulating the virulence of pathogens is likely to be a evolutionally conserved mechanism. Therefore, other host-pathogen systems should be studied to better understand the nature and mechanisms of plant-pathogen interkingdom signaling.

- OryR was originally identified as a LuxR-type solo protein that responds to an unknown rice signal molecule (Gonzalez and Venturi 2013), and we further proved that it plays a key role in the cross-communication between the rice SA/ABA pathways and the *Xoo* QS machinery. Future studies should be focused on exploring the underlying molecular mechanisms and elucidating how OryR regulates ABA-induced swimming. Moreover, it is tempting to extend this line of research to other LuxR solos to examine whether our results are specific for *Xoo* and OryR or, alternatively, represent a conserved virulence strategy shared by multiple bacterial species.

Summary

To fend off various pathogenic microorganisms, plants have evolved a battery of constitutive and inducible defense mechanisms. In the dicot model plant *Arabidopsis thaliana*, the role of small molecule hormones in orchestrating the plant defense network is well established. In contrast, very little is known about the hormone defense networking in rice, a monocot plant model and the most important staple food for more than half the world's population. In view of this knowledge gap, the primary objective of this work was to further our fundamental understanding of hormone defense signaling in monocots, using the interaction between rice and the bacterial leaf blight (BLB) pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) as a model system.

In the first part of this dissertation, we focused on the role of the plant hormone abscisic acid (ABA) in *Xoo* resistance. Through combination of exogenous hormone applications, chemical inhibition of ABA biosynthesis and genetic disruption of downstream ABA signaling, we unmasked ABA as a negative regulator of resistance against *Xoo*. Moreover, infection with *Xoo* was associated with extensive reprogramming of ABA biosynthesis and response genes, and endogenous ABA level suggesting that ABA functions as a virulence factor for *Xoo*. Several lines of evidence indicate that the immune-suppressive effect of ABA is at least in part due to the suppression of salicylic acid (SA)-mediated defenses that normally serve to limit pathogen growth. In contrast, resistance induced by the ABA biosynthesis inhibitor fluridone appears to operate in a SA-independent manner and is likely due to induction of non-specific physiological stress. Collectively, our findings favor a scenario whereby *Xoo* hijacks the rice ABA machinery to cause disease and highlight the importance of ABA and its crosstalk with SA in shaping the outcome of rice-*Xoo* interactions.

In the second part of our study, we characterized the role of the classic growth hormone cytokinin (CK) in the rice-*Xoo* interaction. By exogenous hormone application and bioassays with CK-modified plants, we revealed that CK promotes symptom development and facilitates *Xoo* growth *in planta*. Quantification of endogenous CK content revealed that *Xoo* infection strongly increases CK biosynthesis, which may in turn facilitate *Xoo* invasion. To evaluate the underlying mechanisms, we initially focused on the interaction between CK and SA, as well as its impact on BLB resistance in rice. However, combined molecular, genetic and physiological assays revealed that the CK-induced *Xoo* susceptibility is independent of SA action. In contrast, mounting evidence suggests that CK suppresses BLB resistance by activating Target of Rapamycin, a master regulator of cellular growth. We speculate that CK-induced TOR signaling tilts the plant's innate growth-versus-defense balance towards growth,

thereby reducing the amount of resources available for the plant to mount an effective immune response.

In the third and final part of this work, we tested the hypothesis that plant hormones may directly influence microbial virulence by impinging on the pathogen's quorum sensing (QS) machinery. QS is a cell density-dependent bacterial cell-cell communication mechanism, by which pathogenic bacteria coordinate the expression of virulence traits. In *Xoo*, the diffusible signal factor (DSF)- and the diffusible factor (DF)-mediated QS systems have been shown to orchestrate multiple virulence traits. Our swimming bioassays of the *Xoo* strain XKK12 showed that ABA and SA respectively promote and repress swimming motility. Moreover, SA stimulates the production of exopolysaccharides (EPS) and xanthomonadin, as well as the expression of their biosynthesis genes *gumG* and *xanB2*. Notably, XanB2 catalyzes the biosynthesis of the QS signals DF and 4-HBA, which coordinate the production of xanthomonadin/EPS and coenzyme Q (CoQ), respectively. Interestingly, both DSF, DF and 4-BHA were increased by SA treatment. These results suggest that SA serves as an environmental stimulus to activate the biosynthesis of the three QS signals and enhances the production of xanthomonadin, EPS and CoQ, resulting in the protection of *Xoo* from host defense and facilitation of their invasion. Moreover, in the *oryR* knockout mutant, ABA and SA failed to function while their effects were restored in the *oryR* complementary strain, suggesting the role of LuxR-like 'solo' protein OryR to tap into ABA- and SA-mediated *Xoo* virulence traits. In rice leaf segments, the expression level of the SA marker genes were significantly decreased by exogenous application of DF. Given the structural similarity between DF and SA, we speculate that DF can mimic SA to disturb SA-mediated defense signaling. Taken together, our data indicate that the rice SA and ABA signaling pathways cross-communicate with the DF- and DSF-QS systems in *Xoo* and underscore the importance of bidirectional interkingdom signaling in plant-microbe interactions.

In conclusion, the results denoted in this thesis represent significant progress towards elucidating the role of plant hormones in the rice-*Xoo* pathosystem. On one hand, plant hormones fine-tune rice immunity, whereas on the other hand, they coordinate the virulence strategies of *Xoo*. Moreover, *Xoo* seems to have evolved sophisticated strategies to manipulate the plant's defense signaling circuitry to its own benefit. Such conceptual advances will not only advance our understanding of interkingdom signaling between the pathogen and the host plant, but also may be instrumental in the development of improved crop plants that are better able to withstand multiple stresses.

Samenvatting

Om pathogene micro-organismen af te weren hebben planten een breed spectrum aan constitutieve en induceerbare afweermechanismen ontwikkeld. In de dicotyle modelplant *Arabidopsis thaliana* is de rol van plantenhormonen in de regulatie van deze mechanismen reeds goed beschreven. In graangewassen zoals maïs, tarwe en rogge is echter nog weinig gekend omtrent de functie van hormonen in de natuurlijke immuniteitsrespons. In voorliggend proefschrift hebben we getracht meer inzicht te verwerven in de rol van plantenhormonen in de ziekteresistentie van rijst, de modelplant bij uitstek voor onderzoek naar graangewassen.

In het eerste deel hebben we ons gericht op de rol van het plantenhormoon abscisinezuur (ABA) in de interactie tussen rijst en de bacteriële pathogeen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Uit onze resultaten bleek dat ABA een negatief effect heeft op resistentie tegen *Xoo* en waarschijnlijk fungeert als een virulentiefactor voor de pathogeen. Onze resultaten tonen bovendien aan dat het immuun-onderdrukkende effect van ABA gedeeltelijk veroorzaakt wordt door de onderdrukking van de salicylzuur (SA)-gemedieerde verdediging, welke normaal de groei van de pathogenen beperkt. Resistentie geïnduceerd door de ABA biosynthese inhibitor fluridone bleek dan weer onafhankelijk te zijn van SA en is waarschijnlijk te wijten aan de inductie van niet-specifieke fysiologische stress. Deze bevindingen tonen aan dat ABA een belangrijke rol speelt in het veroorzaken van ziekte door *Xoo* en benadrukken het belang van ABA-SA interacties in de regulatie van rijst-*Xoo* interacties.

Om de rol van plantenhormonen in de immuniteit van rijst verder na te gaan, hebben we in het tweede deel van het onderzoek de rol van cytokinines (CKs) van naderbij bekeken. Door middel van exogene hormoon toedieningen en het gebruik van een CK-deficiënte rijstmutant, hebben we aangetoond dat CKs, net als ABA, verhoogde gevoeligheid voor *Xoo* induceert en een positief effect heeft op de groei van de pathogeen. Uit hormoonmetingen bleek verder dat infectie met *Xoo* zorgt voor een forse stijging in de productie van CK waardoor de pathogeen makkelijker kan binnendringen. Om de onderliggende mechanismen te bestuderen, hebben we ons gericht op de interactie tussen CK en SA, evenals op de mogelijke impact op resistentie in rijst. In tegenstelling tot de gevoeligheid geïnduceerd door ABA, bleek dat CK niet interageert met SA maar inspeelt op de Target of Rapamycin (TOR) pathway. Hierdoor wordt de balans tussen groei en afweerprocessen in de plant verstoord, met verhoogde gevoeligheid van de plant tot gevolg.

In het laatste deel van dit proefschrift hebben we nagegaan of plantenhormonen zoals SA en ABA ook inspelen op de virulentie van de pathogeen, en meer specifiek of deze hormonen de quorum sensing (QS) mechanismen van de bacterie kunnen beïnvloeden. QS is een densiteits-afhankelijk communicatie mechanisme waarmee bacteriën de expressie van hun virulentiegenen kunnen afstemmen op de grootte van de pathogeen populatie. De virulentie van *Xoo* wordt grotendeels bepaald door twee QS systemen die worden gestuurd door verschillende signaalfactoren, met name “diffusible signal factor” (DSF) and “diffusible factor” (DF). Onze bioassays toonden aan dat ABA en SA de beweeglijkheid van de *Xoo* stam XKK12 respectievelijk bevorderen en onderdrukken. Bovendien stimuleert SA de productie van exopolysacchariden (EPS) en xanthomonadines, alsook de expressie van de genen *gumG* en *xanB2*, welke een belangrijke rol spelen in de biosynthese van voornoemde virulentiefactoren. De biosynthese van DF en 4-HBA, welke bepalend zijn voor de productie van respectievelijk xanthomonadin/EPS en CoQ, wordt gekatalyseerd door XanB2. Toediening van SA resulteerde in een toename van DSF, DF en 4-BHA. Deze resultaten suggereren dat, in de aanwezigheid van SA, de biosynthese van de QS signalen DSF, DF en 4-BHA geactiveerd wordt en de productie van xantomonadines, EPS en CoQ toeneemt. Op deze manier kan *Xoo* zich beschermen tegen afweermechanismen van de plant en kan de pathogeen gemakkelijker binnendringen. Onze bevindingen wijzen bovendien op een belangrijke rol van het LuxR-like ‘solo’ protein OryR. Zowel ABA als SA zijn minder effectief in een *oryR* knockout mutant, terwijl het effect van beide hormonen hersteld wordt na complementatie van *oryR*. Verder toonden we aan dat de expressie van SA-afhankelijke genen in de plant afneemt na exogene DF toediening. Gezien de structurele gelijkenis tussen DF en SA, speculeren we dan ook dat DF SA kan nabootsen om zo de SA-gemedieerde afweer van de plant te verstoren. Samenvattend blijkt uit onze gegevens dat de SA en ABA pathways van rijst in nauw contact staan met de DF- en DSF-QS systemen in *Xoo*. Deze bidirectionele signalisatie is belangrijk in de interactie tussen planten en micro-organismen.

In het algemeen kunnen we besluiten dat deze thesis nieuwe inzichten verstrekt in de rol van plantenhormonen in de interactie tussen rijst en *Xoo*. Enerzijds spelen plantenhormonen een rol in de immuniteit van rijst, terwijl ze anderzijds de virulentiestrategieën van de pathogeen beïnvloeden. Bovendien kan *Xoo* ziekte veroorzaken door te interageren met de hormoonwegen van de plant. De bekomen resultaten vormen een uitstekende basis voor verder fundamenteel onderzoek inzake de natuurlijke immuniteitsrespons van rijst en andere graangewassen en kunnen aldus een belangrijke bijdrage leveren in de verdere ontwikkeling van ziekteresistente gewassen.

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- 2010 Advanced Academic English: Writing Skills. Ghent University, Ghent, Belgium.
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Publications

Peer Reviewed

1. **Xu J, Audenaert K, Höfte M*, De Vleesschauwer D*.** (2013) Abscisic acid promotes susceptibility to the rice leaf blight pathogen *Xanthomonas oryzae* pv *oryzae* by suppressing salicylic acid-mediated defenses. *PLOS ONE* 8, e67413
2. **Atalah AB, De Vleesschauwer D, Xu J, Fouquaerta E, Höfte M, Van Dammea EJM*.** (2014) Transcriptional behavior of EUL-related rice lectins towards important abiotic and biotic stresses. *Journal of Plant Physiology*, 171:986-992

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1. **Xu J, Zhou L, Venturi V, He YW, Höfte M*, De Vleesschauwer D*.** Phytohormone-mediated interkingdom signaling shapes the outcome of rice-*Xanthomonas oryzae* pv. *oryzae* interactions, submitted to *BMC Plant Biology*
2. **De Vleesschauwer D, Xu J, Höfte M*.** Making sense of hormone-mediated defense networking: from rice to Arabidopsis (Invited Review), submitted to *Frontiers in Plant Science*

In Preparation

1. **Xu J, Sakakibara H, Höfte M*, De Vleesschauwer D*.** Cytokinin attenuates rice immunity against the leaf blight pathogen *Xanthomonas oryzae* pv *oryzae* by activating the Target-of-Rapamycin (TOR) signaling.

Participation in Conferences and Symposia

With Contribution

- Xu J, Audenaert K, Höfte M*, De Vleesschauwer D*. (2012). Dynamic interplay between abscisic acid, cytokinin and salicylic acid molds innate immunity of rice against the leaf blight pathogen *Xanthomonas oryzae* pv. *oryzae*. 4th Xanthomonas Genomics Conference, July 9-12, 2012, Angers, France. **Post presentation** by Xu J.
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Without Contribution

- 62th International Symposium on Crop Protection, May 18, 2010, Ghent, Belgium.
- 64th International Symposium on Crop Protection, May 22, 2012, Ghent, Belgium.